Dissecting the effects of PTEN on tumour formation and gene expression

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Abstract

The PI3K/PTEN signalling pathway plays an important role in regulation of cell growth, survival, metabolism and migration in many different types of cells. Aberrations in this pathway have implications in metabolic and hyper-proliferative disorders such as diabetes and cancer. PTEN is a tumour suppressor found mutated in many primary and metastatic human cancers. It has lipid and protein phosphatase activities, which are both required to inhibit cellular invasion and mediate most of its largest effects on gene expression. PTEN can antagonize the PI3K/AKT pathway by dephosphorylating phosphatidylinositol 3,4,5 trisphosphate (PIP_3) thereby acting as tumour suppressor. Most of PTEN's tumour suppressor activities have been attributed to its ability to regulate signalling downstream of AKT. However, our recent data in glioma cells have shown that two PTEN mutants, PTEN Y138L and PTEN R308C, can regulate AKT phosphorylation and signalling downstream but fail to regulate processes such as cell invasion and epithelial cell architecture. These findings suggest that in some circumstances the regulation not of AKT, but of signalling mechanisms that control invasion can correlate with PTEN-mediated tumour suppression. In order to understand how PTEN could regulate tumorigenesis, two new transgenic knock in mice lines were developed expressing Pten Y138L and Pten R308C mutants. The Y138L mutation is embryonic lethal and homozygous embryos die at day 9.5 of pregnancy. Interestingly, heterozygous PTEN Y138L mice develop tumour as early as 5 months and about half develop tumours before 18 months of age. R308C heterozygous and homozygous mice are both viable instead, but data suggest that the mutation may also be tumorigenic. Furthermore, to understand how PTEN WT and PTEN Y138L could regulate gene expression, I performed luciferase promoter reporter assays. From these first experiments, significant effects of PTEN WT or PTEN Y138L on gene expression were not detected. Together, these studies help us understand how PTEN suppresses tumour formation in vivo and regulate gene expression.

To my father

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Chapter 1

1. Introduction

1.1 General introduction

Processes that regulate the life of the cells and in particular those that influence cell growth and proliferation are properly regulated through highly complex and structured signalling systems. Indeed, for normal functioning and homeostasis, all multicellular organisms need a fine balance between cell growth and cell death. An illustration of this is that defects in the control of cell proliferation can lead to hyper-proliferative diseases such as cancer.

1.1.1 Mechanism of tumorigenesis

Balance between cell death and proliferation, and balanced regulatory exchanges between stromal and epithelial cells are both vital components of the dynamic equilibrium of the internal environment of the human body. Loss of this balance can lead to cancer: an uncontrolled growth of cells (often benign), which can eventually invade other tissues by migrating from their original site to proliferate elsewhere (malignant). Tumorigenesis is a process of clonal evolution that is initiated from single cells within otherwise histologically normal tissue. Cells in normal tissue continually undergo mutation; some of those may give the cell a proliferation advantage over the other cells in the population. In the carcinogenesis process, cancer cells have been shown to accumulate multiple mutations that are believed to drive their transformation from normal to tumour cells. Pro-tumorigenic mutations affect two classes of genes: oncogenes and tumour suppressor genes (TSGs).

Oncogenes are defined as genes in which mutations affect tumour formation through gain of function, which can either constitutively activate or lead to an increased expression of the oncoproteins, resulting in amplification of their functions. The products of oncogenes include transcription factors, chromatin re-modellers, growth factors, growth factor receptors, signal transducers, and apoptosis regulators Croce (2008). Many of these oncogenes are targets for drug development. Small molecule inhibitors of some of these oncoproteins have been approved for use and many of them are in clinical trials.

Mutations in TSGs lead to loss of activity and relieve their inhibition on cell growth and proliferation. The first tumour suppressor to be discovered was Retinoblastoma (Rb) protein by Alfred Knudson in 1971. He proposed the two hit model for loss of function of TSGs which suggested that they undergo biallelic inactivation. The inactivation of the first allele could either be germ line or somatic. The second mutation is always somatic. The occurrence of the second mutation is often detected as an experimental loss of heterozygosis (LOH) (Lee & Muller 2010).

Although supported in some instances by a wealth of data, the two hit model however does not apply to all tumour suppressor genes. Only some TSGs are truly recessive. Loss of a single copy of some TSGs, also termed as haploinsufficiency, may be sufficient to cause a phenotype. Examples of haplo-insufficient genes include p53, PTEN, SMAD4 and LKB1 (Berger, Knudson et al. 2011), (Berger and Pandolfi 2011).

In almost all cancers, a single mutation alone is not sufficient for tumour progression, except done for cancer types in which very few, or none, mutations are identified, i.e. medulloblastoma and some leukemias. Cancer cells undergo various genetic and epigenetic changes and acquire biological capabilities in a multistep tumour development process which allow them to be tumourigenic and ultimately malignant. Robert Weinberg and colleagues have classified these acquired capabilities into eight categories and these are called as the hallmarks of cancer.

One of the early steps in the initiation of most tumours is a mutation in proteins such as p53 and telomerase, which confers immortality: the ability for continuous cell growth and limitless replication. Once the cell is capable of uncontrolled proliferation, it needs to rewire its regulatory pathways for sustaining the chronic proliferation. This is accomplished by amplification of oncogenes involved in growth factor signalling. Examples include receptor tyrosine kinases like the EGFR, downstream kinases like PI 3- kinases, AKT, mTOR etc.

In addition, the cell needs to evade growth suppression by loss of function of tumour suppressor genes, and resist cell death by mutations of the genes responsible for apoptosis. The primary tumour undergoes vascularisation by angiogenesis and it can form metastasis of that are initiated by cells escaping the primary tumour into the circulation through these blood vessels feeding the tumour (Hanahan & Weinberg, 2011). It seems reliable that many and perhaps all cancer cells develop also the capability to modify, or reprogram, cellular metabolism in order to support neoplastic proliferation and to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells [T cell subtype]. Currently, there have been drugs developed, and some of them are already in clinical trials, which interfere with these capabilities of the tumours required for its growth, progression and escape from immune system and programmed death (**Figure 1.1**).(Hanahan and Weinberg 2011).

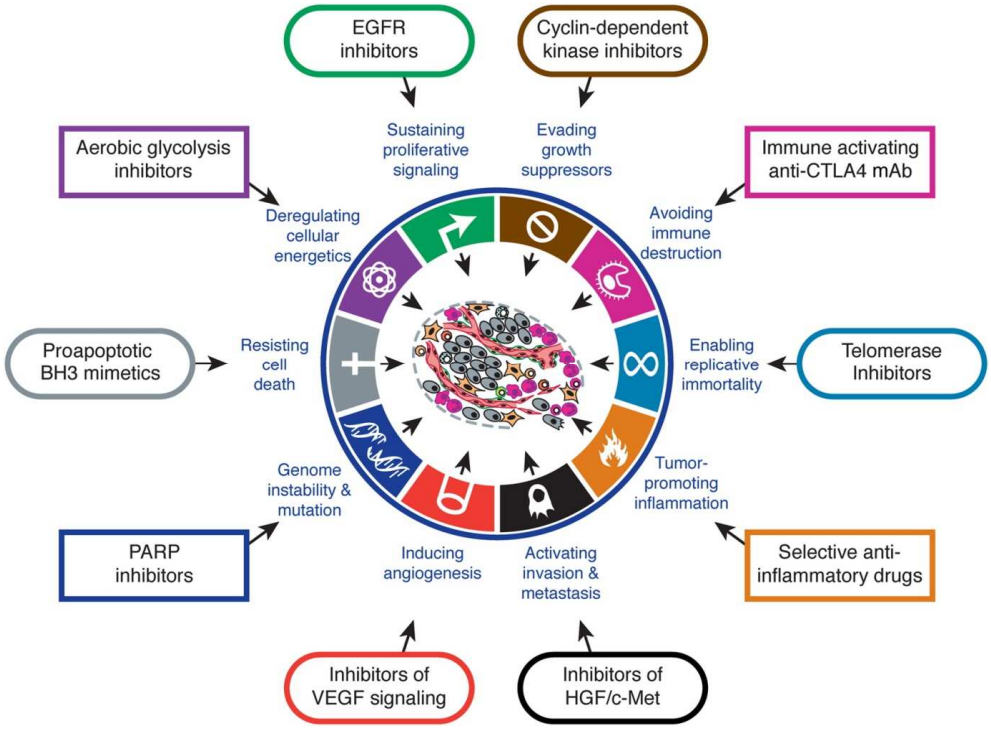

Figure 1.1| Therapeutic targeting of the Hallmarks of Cancer. Drugs that interfere with each of the acquired capabilities necessary for tumour growth and progression have been developed and are in clinical trials or in some cases approved for clinical use in treating certain forms of human cancer. (Figure from Hanahan & Weinberg, 2011)

Fearon and Volgestein, using colon carcinogenesis as an example, described tumourigenesis, adopting a linear progression model, according to which malignant tumours evolve from pre-existing and less aggressive benign tumours (Fearon and Vogelstein 1990). This classical model of carcinogenesis can be described as a three-step process including initiation, promotion and progression. Exposure of cells to endogenous and exogenous genotoxic agents (reactive oxygen species (ROS), double strand breaks due to UV light, and other reactive metabolites), represents the first step of the complicated machinery, resulting in DNA mutations (point mutations, deletions, insertions, amplifications, chromosomal translocations) and chromosome instability. In particular, changes in genes that themselves regulate the repair of DNA damage contribute significantly to tumour promotion and progression (Iida, Shimada et al. 2013). Beside genetic mutations, non genotoxic agents like diet, obesity and environment are also considered as modulators of cancer risk.

Tumours often possess genetically different clones that arise from initial transformed cells through further genetic alterations. This heterogeneity contributes to differences in clinical behaviour and responses to treatment of tumours of the same diagnostic type. These populations can differ in sensitivity to chemotherapy, radiotherapy, and other treatments, making clinical management difficult. For these reasons, the initiating steps in the development of cancer are of considerable clinical importance and are a priority in the development of rational cancer treatment.

The focus of this Master of Philosophy project is the PI 3-kinase-PTEN signalling network whose importance in human cancer has been shown by its activation in many, and probably most, tumours.

1.2 The PI3-kinases signalling Pathway

Phosphoinositide 3-kinases (PI 3-kinases) are the most widely studied family of PI kinases. As evident from their name, they can phosphorylate the 3rd hydroxyl position of the inositol ring of phosphoinositide lipids. Phosphatidylinositol and its derivatives form around 2-10% of total cellular lipids in eukaryotic cells. They are amphipathic molecules consisting of a glycerol backbone, two fatty acid chains and a polar phosphate group substituted with an inositol head group. The inositol ring of phosphatidylinositol has 5 free hydroxyl groups, 3 of which have been found to be phosphorylated, generating a set of lipids that collectively are called phosphoinositides (PIs). The phosphoinositides are much less abundant than the precursor phosphatidylinositol (Mulgrew-Nesbitt et al, 2006). The different phosphoinositides found in cells are PtdIns3P, PtdIns4P, PtdIns5P, PtdIns(3,5)P_2, PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. Phosphoinositides have many functions. They can be metabolised by different enzymes such as kinases, phosphatases and lipases to generate secondary messengers that play roles in several signalling pathways. Phosphoinositides can bind to proteins and affect their localisation (Balla et al, 2009).

PI 3-kinases have been broadly classified into three classes; Class-I, II and III, based on their structure and lipid substrate preference (Domin, Pages et al. 1997); (Cantley et al, 2002); (Chalhoub, Zhu et al. 2009); (Vanhaesebroeck, Vogt et al. 2010). In particular Class-I PI 3-kinases are the most studied of all PI Kinases and use PtdIns(4,5)P_2 as a cellular substrate to give PtdIns(3,4,5)P_3 and are activated by receptor tyrosine kinases, cytokine receptors and also some G Protein Coupled Receptors (GPCR) (Guillermet-Guibert et al, 2008). PtdIns(3,4,5)P_3 then mediates its effects on cellular behaviour through proteins that are able to selectively bind to the lipid. The best recognised PtdIns(3,4,5)P_3 effector proteins bind to the lipid through pleckstrin homology (PH) domains, and include Ser/Thr kinases such as AKT and PDK1, Tyrosine kinases, adaptor proteins and regulators (GAPs and GEFs) of small GTPases of the Rac and ARF families. Binding of these

proteins to the PtdIns(3,4,5)P_3 in the membrane triggers a cascade of downstream signalling events (Engelman, Luo et al. 2006).

1.2.1 The PI-3Kinase/AKT pathway

One of the most studied effectors of the Class-I PI 3-kinases is AKT. It is a Ser/Thr protein kinase which was discovered based on sequence homology to v-AKT, the transforming oncogene of the AKT 8 retrovirus Staal (1987). Mammalian cells express three different isoforms of AKT: AKT 1, AKT 2 and AKT 3. The AKT kinases have a PH domain which can bind to 3-phosphoinositides such as PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. The mechanism of upstream activation of all three isoforms appears the same. However they may have different downstream effectors. AKT 1 and AKT 2 are more widely expressed than AKT 3.

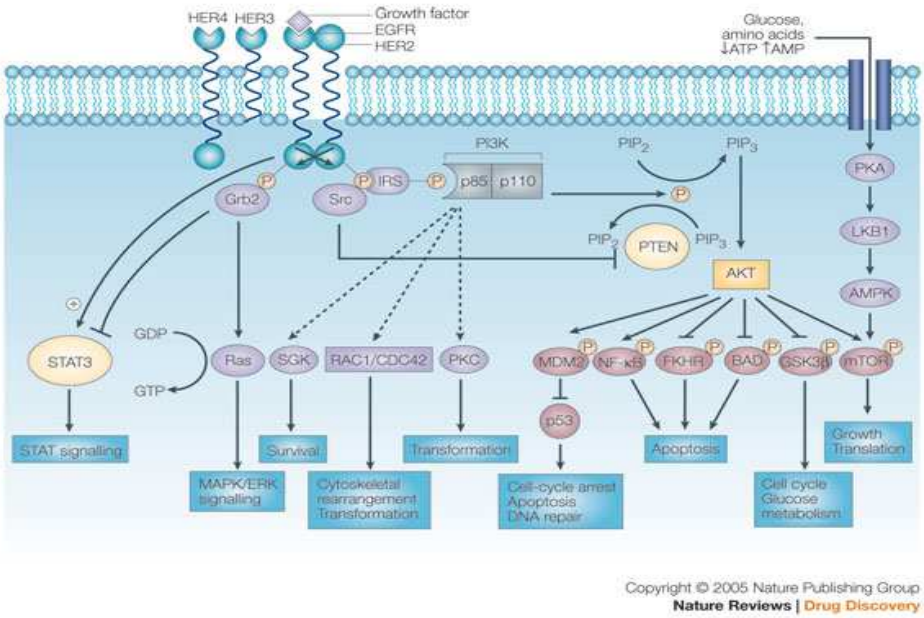

Figure 1.2| The PI3K/AKT and related pathways. The PI3K/AKT and related pathways are important in internalizing the effects of external growth factors and of membrane tyrosine kinases. Activation of membrane kinases including epidermal growth factor receptor (EGFR) by external growth factors initiates receptor dimerization and subsequent events to activate these intracellular pathways. AKT is activated downstream of PI3K and has multiple targets. It is involved in regulation of growth, apoptosis, glucose metabolism, cell cycle and DNA repair (Hennessy 2005).

The AKT proteins are approximately 65kDa proteins. They have a canonical N-terminal PH domain, which facilitates binding to phosphoinositides, a central kinase domain and a C-terminal hydrophobic motif (HM). The kinase domain and HM of AKT bears high sequence homology to other members of the AGC kinases.

PtdIns(3,4,5)P_3 binding to the PH domain of AKT brings about a conformational change in AKT exposing two critical phosphorylation sites. Thr-308, present in the activation loop of the kinase domain is phosphorylated by PDK1 and Ser-473 in a hydrophobic motif is phosphorylated by the mTORC2 (mTOR-Rictor) complex. Phosphorylation at these sites stably activates the kinase activity of AKT which can then phosphorylate downstream effector proteins in multiple locations and control processes such as protein synthesis, survival, proliferation, glucose metabolism etc. The minimal substrate phosphorylation motif for AKT has been identified as R-X-R-X-X-S/T- β, where S/T represents the serine or threonine residue that becomes phosphorylated and β represents a large hydrophobic residue (Alessi, Caudwell et al. 1996). A large number of candidate are reported, however not all have been characterised in detail. It has been demonstrated that AKT has a critical role in promoting cell survival, and it fulfils this function by blocking directly the expression of proapoptotic Bcl-2 homology domain 3 (BH3)-only proteins such as BAD, which usually inactivates pro-survival Bcl-2 family members, or through effects on transcription factors such as FOXO and p53. Indeed, FOXO (1, 3a, and 4), phosphorylated by AKT, is exported from the nucleus by 14-3-3 proteins-binding with block of FOXO-mediated transcription of target genes (including FasL and Bim), which promote apoptosis, cell-cycle arrest, and control many metabolic processes (Brunet, Bonni et al. 1999); Dijkers, Birkenkamp et al. (2002). In contrast, phosphorylation of E3 ubiquitin ligase MDM2 by AKT promotes its translocation to the nucleus where it triggers p53 degradation and thus cell survival (Mayo, Dixon et al. 2002). AKT can inhibit apoptosis through effects on several other targets such as inhibition of GSK3 and JNK/p38 apoptotic signals, or activation of NFKB 45 survival signalling (Cross, Alessi et al. 1995); (Kim, Zhou et al. 2006); (Maurer, Charvet et al. 2006); (Ozes, (Mayo

et al. 1999), however their functions are less clear. An important downstream substrate is the mammalian target of rapamycin kinase mTOR, a critical regulator of cell growth. mTOR complex 1 (mTORC1) or its downstream substrates, S6K1 and 4E-BP1(eukaryotic initiation factor 4E (eIF4E)-binding protein 1), is phosphorylated and activated by AKT. AKT can regulate mTORC1 also through inhibition of the tumour suppressor TSC2 (tuberous sclerosis complex 2), a negative regulator of the complex. In addition, phosphorylation of TSC1/2 complex, inhibitor of Ras-related small G protein Rheb, relieves its inhibition of Rheb activity, leading to activation of the rapamycin-sensitive mTOR complex (mTORC1) (Gao and Pan 2001); (Potter, Pedraza et al. 2002); (Sekulic, Hudson et al. 2000); (Tapon, Ito et al. 2001). Activated AKT modulates also the function of multiple downstream targets involved in the regulation of cell proliferation, glycogen metabolism and protein synthesis. The AKT kinases are strongly activated by insulin and of the three AKT isoforms, AKT 2 is most strongly associated with glucose homeostasis. Initial studies using knock-out mice showed that AKT 2 null mice were glucose intolerant and insulin resistant which led to diabetes and pancreatic β cell failure. This was due to defective glucose uptake on insulin stimulation by the muscle and adipose tissue. AKT 1 and AKT 3 null mice had normal glucose metabolism. The mechanism for this was reported to be AKT 2 dependent regulation of the Glut4 glucose transport protein. On insulin stimulation, Glut4 containing intracellular vesicles are rapidly translocated to the plasma membrane where the vesicles fuse with the membrane and insert Glut4 in cell the membrane. Once at the membrane, Glut4 allows glucose uptake. Translocation of Glut4 requires activity of Rab proteins. AKT plays a major role in this process by phosphorylating and inhibiting the GAPs for the Rab proteins. This releases the GAP inhibition of the respective Rab proteins and this activates them facilitating Glut4 translocation. One such GAP regulated by AKT is the AS160 (or TBC1D4) protein (Miinea, Sano et al. 2005). AKT can phosphorylate AS160 at 5 residues (Ser- 318, Ser-570, Ser-588, Thr-642 and Thr-571) in response to insulin stimulation, and inhibit its GAP activity. GSK3 enzymes are known to regulate Glycogen synthase, which converts glucose to glycogen. AKT can phosphorylate GSK3α and β at the Serine 9 and 21 respectively and inhibit

the kinase activity of GSK3 and thus inhibits glycogen synthesis. Inhibition of GSK3B is also known to promote sterol regulatory element binding proteins, which can induce transcription of genes required for cholesterol and fatty acid biosynthesis. Almost 100 substrates have been proposed for GSK3 that suggests that it can regulate multiple cellular processes (Sutherland, 2011) (**Figure 1.2**).

The importance of this pathway is assessed also by studies conducted in other organisms, as yeast, Caenorhabditis elegans and Drosophila. Although class I phosphatidylinositol 3-kinases (PI3Ks) have not been found in yeast, there is some evidence of pathway conservation. Homologues of other members of the PI3K pathway have been described, including two PDK homologues, named Pkh1 and Pkh2 (Pkb-activating kinase homologues 1 and 2), which are essential for viability and activate human AKT1 in vitro112, and two Tor proteins that regulate biosynthesis. In *C. elegans*, the PI3K (or Age1) signalling pathway regulates adult longevity and dauer diapause, a larval developmental stage that is induced by unfavourable environmental conditions. The *C. elegans* insulin receptor Daf2 lies upstream of Age1 and signals through Akt1 and Akt2 in a process that also requires the activity of Pdk1. The homology of the pathway extends to downstream targets of Akt, such as the forkhead transcription factor Daf16 and to negative regulators, such as Daf18, the C. elegans PTEN homologue. *Drosophila* homologues exist for all characterized members of the PI3K pathway, from insulin-like peptides to downstream effectors of Akt. Genetic studies have implicated PI3K signalling in cell growth and proliferation. The fly phenotype of loss-of-function mutants in the pathway is a decrease in organ and body size owing to changes in cell size and number. Conversely, mutations in the negative regulator Pten cause an increase in cell size and cell proliferation (Vivanco and Sawyers 2002).

The Class I PI 3-kinase/ AKT signalling system is one of the most important that mammalian cells use to control cell growth and proliferation. Indeed aberrations in this pathway have implications in metabolic and hyper-proliferative disorders such as diabetes and cancer. Specifically, functions of the many members of the PI 3-kinase signalling pathway are found to be

altered in many types of cancers. There is frequent amplification of the function of proteins such as RTKs, PI 3-kinase and AKT and loss of function of negative regulators, such as PTEN and INPP4B in cancer. Thus PI 3-kinase pathway effector proteins are a common target for cancer therapy. Various small molecule inhibitors for RTKs, PI 3-kinases, AKT and downstream effectors such as mTOR are being used as drugs for treating cancers with amplified PI 3-kinase signalling (McNamara and Degterev 2011), (Vanhaesebroeck, Guillermet-Guibert et al. 2010); (Wong, Engelman et al. 2010); (Zhao and Vogt 2008).

There are several inhibitory component of the PI3K pathway (phosphatases, E3 ligases etc), but it appear the most frequently changed in cancer is the PIP_3 phosphatase PTEN. The PI 3-Kinase / AKT pathway is mainly antagonized by PTEN that is a soluble, largely cytosolic protein that removes PIP_3 by acting as an interfacial phosphatase at the cell membrane.

1.3 PTEN

Homozygous deletion of regions of human chromosome 10q23 is a common event found in many human cancers especially glioblastoma multiforme. Mapping of these deletions led to the discovery of PTEN as a putative tumour suppressor (Li, Yen et al. 1997); (Steck, Pershouse et al. 1997). Since then, PTEN mutations have been reported in a multitude of human cancers (Ali, Schriml et al. 1999); (Bonneau and Longy 2000). Mouse models have also been used to confirm the effects of PTEN loss and mutation on tumour development in many tissues. Biochemical studies have explained the functions of PTEN. All of these have improved our understanding of the tumour suppressor functions of PTEN and are discussed below in detail.

1.3.1 PTEN structure

The predominant form of human PTEN is a 403 amino acid cytosolic protein. It can be structurally divided mainly into an N-terminal phosphatase domain and a C-terminal C2 domain. It also has a short 7 amino acid N-terminal sequence, also called PtdIns(4,5)P_2 binding motif (PBM), which, along with the extreme N-terminus of the phosphatase domain is required for binding to the membrane lipid PtdIns(4,5)P_2 (Walker, Leslie et al. 2004). Finally, there is a C-terminal tail with limited structure consisting of several phosphorylation sites. At its extreme C-terminus PTEN has an ITKV motif which is responsible for interaction with PDZ domains and is called the PDZ binding motif (PDZBM). A crystal structure of PTEN was reported in 1999 (Lee, Yang et al. 1999). (**Figure 1.3**) PTEN consists of three unstructured regions or loops which are sensitive to proteolytic digestion and were not included in the crystal structure. These include the N-terminal PBM (residues 1-7), C terminal tail (residues 354- 403, 50 amino acids) and a 24 amino acid internal loop in the C2 domain (residues 286-309, named the DLoop by Spinelli et al, unpublished data).

Although PTEN belongs to the PTP family of Dual Specifity Phosphatases (DSPs), that dephosphorylate both phospho-tyrosine and phospho-serine/ threonine substrates. It contains the signature catalytic motif HCXXGXXR of the family (also called the P loop) and it bears very little sequence homology with most other members of this family outside this catalytic motif. The phosphatase domain consists of five central β- strands flanked by two α-helices on one side and five α-helices on the other. The catalytic pocket of PTEN consists of the P-loop and WPD loop residues, which play a role in catalysis, substrate recognition and conformation of the catalytic pocket.

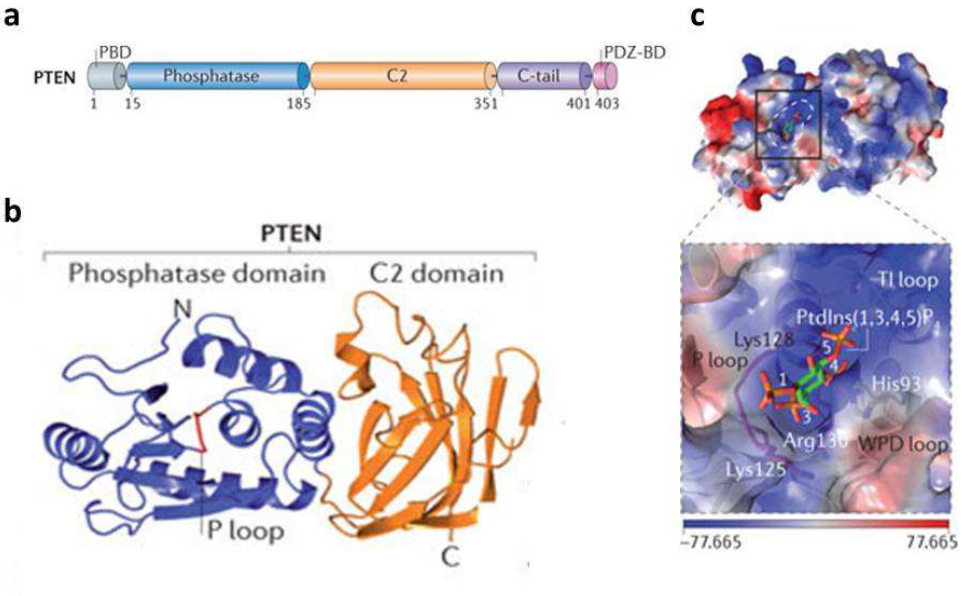

Figure 1.3| a. The domain structure of phosphatase and tensin homologue (PTEN). PTEN is a 403 amino acid protein that is composed of five functional domains: a phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2)-binding domain (PBD), a phosphatase domain, a C2 domain, a carboxy-terminal tail and a PDZ-binding domain. **b**. Overview of the PTEN protein structure. The structure of the PTEN phosphatase domain (dark blue) consists of a central five-stranded β-sheet that packs with two α-helices on one side and four on the other. The C-terminal 170 amino acids of PTEN fold into a β-sandwich, containing two antiparallel β-sheets with two short α-helices intervening between the strands (orange). **c.** Active sites of PTEN. The larger width of and the positive charge in the PTEN pocket are important for the accommodation of phosphoinositide substrates, as demonstrated here by Ins(1,3,4,5)P_4. (Figure adapted from Song 2012)

1.3.2 PTEN as lipid and protein phosphatase

PTEN has the signature catalytic motif of the PTP family of dual specificity phosphatases. This sub-family get their name from their ability, shared by PTEN, to dephosphorylate both phospho-tyrosine and phospho-serine/ threonine substrates.

When first cloned, PTEN was classified as a protein phosphatase since it contained the signature motif HCXXGXXR**S/T** found in protein tyrosine phosphatase (PTPs) and dual-specificity phosphatase (DSPs) (Li, Yen et al. 1997); (Myers and Tonks 1997); (Steck, Pershouse et al. 1997); (Tonks and Neel 1996); (Yuvaniyama, Denu et al. 1996). Within this motif, the conserved active site cysteine 124, located at the base of the catalytic pocket, is required for the catalytic activity of PTEN, as its sulfhydryl group functions as a nucleophile that attacks the phosphorous atom of its substrate with the formation of a thiophosphate-enzyme intermediate (Myers and Tonks 1997); (Zhou, Tarmin et al. 1994). Indeed, mutation of cysteine 124 to serine in PTEN resulted in a complete loss of its phosphatase activity and in the ability of PTEN to control cellular growth in PTEN-deficient tumour cell (Furnari, Lin et al. 1997), (Furnari, Huang et al. 1998); (Li and Sun 1997). PTEN exhibits a robust phosphatase activity against the highly acidic and artificial random copolymer of glutamate and tyrosine, (polyGlu4,Tyr1). Several studies have observed changes in PtdIns(3,4,5)P_3 levels when PTEN was overexpressed in different cell lines including HEK293T cells and glioma cells, with alteration of the signalling downstream of PI3-Kinase, whereas PTEN C124S, unable to dephosphorylate protein substrates, was also unable to dephosphorylate PtdIns(3,4,5)P_3 (Maehama and Dixon 1998); (Myers et al., 1998). Characterisation of the lipid phosphatase activity of PTEN demonstrated that it can hydrolyse PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, showing high specificity for the latter even when compared with its water-soluble head group, Ins(1,3,4,5)P_4 (McConnachie, Pass et al. 2003); (Myers, Pass et al. 1998). Taken together, these data confirmed PtdIns(3,4,5)P_3 as a physiological lipid substrate for PTEN.

Recently, it has been demonstrated that minimal part of the total amount of PTEN in the cell exists in a dimeric conformation. Dimeric PTEN complexes are more active than PTEN monomer in dephosphorylating PIP3 and regulating PI3K signalling. In particular, Papa et al showed that while disrupting PTEN activity in cis, PTEN C124S and PTEN G129E inhibit the WT protein function in trans in a dominant- negative manner as a result of heterodimerization. In turn, this reduced Pten lipid-phosphatase activity leads to Akt hyperactivation and increased tumourigenesis in the mouse. Moreover, it has previously been published that phosphorylation of the PTEN tail is known to produce an inactive form of the enzyme in a closed conformation (Leslie and Foti 2011). In agreement, Papa et al. provide evidence that dephosphorylation of the PTEN-tail, while favouring a more open conformation, also allows subsequent dimerization and, possibly, oligomerization, in view of the multiple interfaces found to mediate the PTEN-PTEN interaction (Papa et al. 2014).

In contrast to its lipid phosphatase activity, PTEN has a weaker activity was detected when a PTEN against the common protein tyrosine phosphatase substrates, and complete ablation of PTEN catalytic activity was observed when the mutant PTEN C124S was used. PTEN favours acidic peptides but will not dephosphorylate all acidic phospho peptides which shows that even PTEN's protein phosphatase activity has some degree of substrate specificity (Myers and Tonks 1997). Various studies have suggested that PTEN's protein phosphatase activity has important biological consequences. While some of these effects have been proposed to require PTEN's ability to dephosphorylate heterologous protein substrates, the others are indirect consequences of PTEN's protein phosphatase activity. These have been discussed below. The importance of PTEN's protein phosphatase activity over its lipid phosphatase activity in these processes has been implicated in studies using the PTEN mutant G129E. This PTEN mutation was found in two Cowden disease families (Liaw, Marsh et al. 1997). The Glycine 129 residue is present at the bottom of the catalytic pocket of PTEN (Lee, Yang et al. 1999). Mutation of this residue to glutamic acid reduces the size of the pocket. This selectively impairs the ability of the mutant PTEN enzyme to

metabolise the lipid substrate PtdIns(3,4,5)P_3 and its soluble head group Inositol (1,3,4,5)-tetrakis phosphate (IP4). However, this mutant retains the ability to metabolise the peptide substrate PolyGluTyr(P) (Furnari, Huang et al. 1998); (Myers and Tonks 1997), (Myers, Pass et al. 1998). Its activity against phosphorylated serine/threonine substrates has not been tested. The low protein phosphatase activity measured for PTEN compared to other PTPs, suggested that PTEN might be active against non-protein substrates. Tamura and colleagues showed that PTEN inhibited fibroblast migration, and they correlated this effect to its association with the focal adhesion kinase (FAK), which was shown to be dephosphorylated by PTEN. However, later findings reported that FAK was not dephosphorylated when a protein phosphatase active, Cowden-associated mutation, PTEN G129E, was used. Therefore, this implied the presence of other substrates for PTEN (Myers and Tonks 1997); (Tamura, Tamura et al. 1998). Therefore, the finding of PTEN as a PtdIns(3,4,5)P_3 phosphatase implied that deletion or mutations of PTEN led to the activation of PI3-kinase pathway. several substrates have been proposed for PTEN such as FAK, Shc, IRS-1, Fyn, p85β, PDGFR, β-catenin, 5 H2TC, Par3, CREB, Cyclin D1 and PTEN itself. (Tamura et al. 1998) (Gu, Zhang et al. 2011), (Dey, Crosswell et al. (2008); (He, Ingram et al. 2010); (Ramachandran, Barria et al. 2009); Gu, Zhang et al. 2011);Vogelmann, Nguyen-Tat et al. 2005), (Tibarewal, Zilidis et al. 2012). In particular PTEN appears to dephosphorylate itself at Threonine 366 being able to autoregulate its lipid phosphatase activity, in fact mutation of this site makes lipid phosphatase activity sufficient for PTEN to inhibit invasion (Tibarewal, Zilidis et al. 2012) and control epithelial morphology (Berglund et al, 2013). The role of PTEN's protein phosphatase activity has also been implicated in inhibition of cell migration and epithelial to mesenchymal transition (EMT) (Leslie, Yang et al. 2007); (Tamura et al. 1998). A summarized illustration of PTEN as lipid and protein phosphatase is shown in Figure 1.4

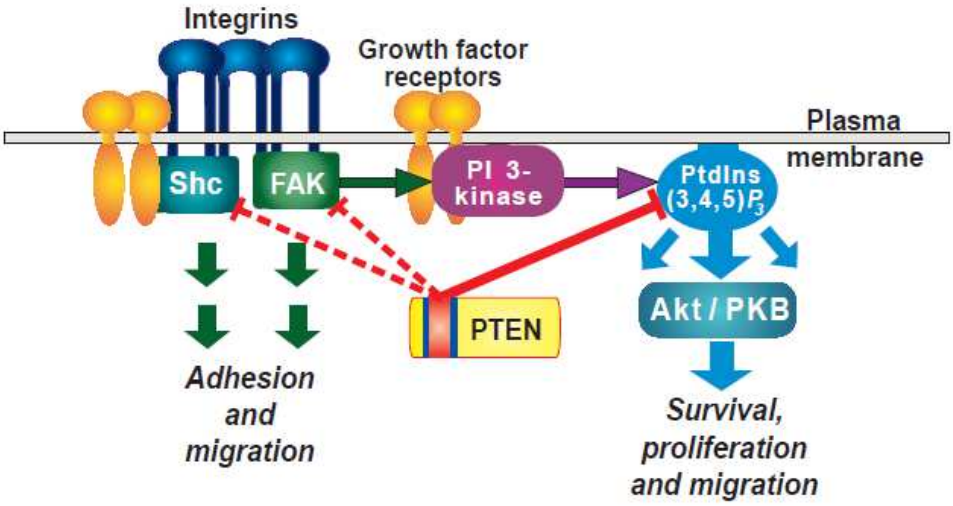

Figure 1.4| Reported sites of action of PTEN. Extracellular interactions trigger signalling from integrins and growth factor receptors. The major function of PTEN is the downregulation of the PI3-Kinase product PtdIns(3,4,5)P_3, which regulates Akt and complex downstream pathways affecting cell growth, survival and migration. In addition, PTEN has weak protein tyrosine phosphatase activity, which may target focal adhesion kinase (FAK) and Shc, and thereby modulate other pathways. The phosphatase domain of PTEN (red) may dephosphorylate and downregulate (red lines) substrate molecules (Figure from Yamada and Araki (2001).

1.3.3 Mutational spectra of PTEN

PTEN is constitutively expressed in normal cells but under pathological conditions, PTEN levels can be dramatically reduced, indeed it is been found mutated in many primary and metastatic human cancers. Homozygous deletion of regions of human chromosome 10q23 is also a common event found in human tumours especially glioblastoma multiforme (Ali, Schriml et al. 1999); (Bonneau and Longy 2000).

PTEN mutations can either be germline or somatic. Germline mutations of PTEN can lead to the PTEN Hamartoma syndrome (PHTS) and are also linked to autism, whereas somatic mutations occur in many sporadic cancers.

PHTS are characterised by formation of histologically distinct subtype of benign tumours called hamartomas. PHTS can be further classified into Cowden syndrome, Bannayan Riley Ruvalcaba syndrome, Lhermitte Duclos disease and Proteus syndrome. The most common of these is Cowden Syndrome. It is characterised by hamartomas of skin, breast, thyroid, endometrium, gastrointestinal tract and central nervous system, and a predisposition to malignancies of breast (25-50% of affected women) and thyroid (3-10% of all affected patients). Table 1.9 shows the frequency of PTEN mutations and clinical manifestations in the above mentioned syndromes. PTEN germline mutations also lead to Autism spectrum disorder (ASD) with macrocephaly. Germline mutations of PTEN have also been found in patients diagnosed as having GI tract hamartoma syndromes such as Peutz Jeghers Syndrome and Juvenile Polyposis Syndrome (Ali, Schriml et al. 1999); (Blumenthal and Dennis 2008).

Homozygous mutations and loss of heterozygosity of PTEN are common features of many cancers as shown in Table 1.1. Somatic loss of PTEN function through mutation, deletion and promoter methylation is a common event in many human cancers. The COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/) provides a comprehensive list of somatic mutations of PTEN found in human cancers (Chalhoub and Baker 2009); (Wong, Engelman et al. 2010). A histogram of the frequency of

mutations for individual amino acids of PTEN is shown in Figure 1.5. Highlighted in this figure are the mutational hotspots in PTEN.

Tissue	*PTEN* alteration in human cancer	Neoplasms and tumours in PHTS	Tumours in *Pten*$^{+/-}$ mice
Breast	Mutation <5%, LOH 40%, promoter methylation 50% and loss of expression ~40%	25–50% lifetime risk for women	Yes
Endometrium	Mutation 35–50%	Yes	Yes
Thyroid	Homozygous deletion <10%, promoter methylation >50%, and rearrangement in most papillary thyroid carcinomas	Yes	Late onset and low frequency
Prostate	Frequent LOH and miR-22 and miR-106b–25 cluster overexpression	NR	Late onset
Leukaemia or lymphoma	Deletion 10% of T-ALL and 27% mutation in T-ALL	NR	Lymphoma and radiation decreases latency
Glioma	LOH >70%, mutation 44% (coincident with LOH) and miR-26a amplification	Dysplastic gangliocytoma of the cerebellum in LD	NR
Melanoma	LOH 30–60%, mutation 10–20% (metastases) and >50% frequent promoter methylation in patients with XP	NR	NR
Lung cancer	Mutation infrequent, promoter methylation frequent, miR-21 upregulation 74% and loss of PTEN 74%	Occasional	NR
Liver	Mutation <5%, PTEN expression lost in 12% and PTEN expression lost in HepC HCC	NR	Infrequent
Bladder	LOH 23%, homozygous deletion 6%, mutation 23% (late stage) and decreased or absent PTEN expression 53%	NR	NR
Kidney	LOH 25%	NR	NR
Pancreas	Altered localization common	NR	NR
Adrenal pheochromocytoma	LOH more common in malignant than in benign tumours	NR	Yes
Colon and intestine	Up to 18% mutated and up to 19% LOH depending on tumour type	Yes and benign polyps in >90%	Hyperplastic changes

HepC HCC, hepatitis C-positive hepatocellular carcinoma; LD, Lhermitte–Duclos syndrome
PHTS, PTEN hamartoma tumour syndromes; T-ALL, T cell acute lymphocytic leukaemia; XP,
latency or increased tumour stage in the presence of these additional alterations.

Table 1.1| Summary of evidence for PTEN and Pten alteration in specific cancers, by tissue (Table adapted from Hollander, Blumenthal et al. 2011)

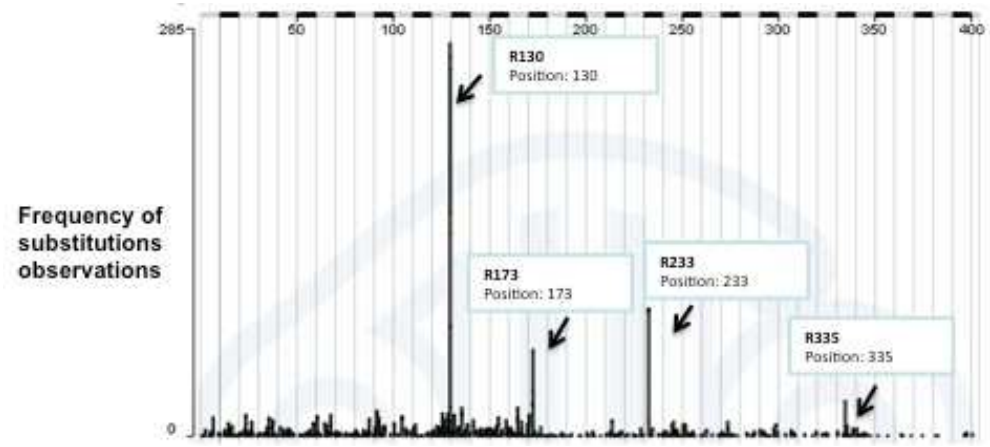

Figure 1.5| Somatic mutations of PTEN found in human cancers. The histogram shows the frequency of single amino acid mutations of PTEN from the COSMIC database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). The amino acids are shown along the X□axis and the number of mutations found is shown on the Y□axis. The mutational hot□spots have been indicated in the figure. It is noteworthy that all these amino acids are arginines and have the CGN codon (R130□CGA, 173□CGC, R233□CGA and R335□CGA). Of the 20 arginines in PTEN, 6 have the CGN codon (above mentioned, R142 and R308) and the remaining have AGN codons.

1.3.4 PTEN mouse models

Mouse models of human cancer have played a vital role in understanding tumourigenesis and answering experimental questions that other systems cannot address. Advances continue to be made that allow better understanding of the mechanisms of tumour development, and therefore the identification of better therapeutic and diagnostic strategies. Many research groups have used mice models to reconstruct in vivo the effects of PTEN loss and mutations on tumour development and cancer progression.

To examine the role of PTEN in ontogenesis and tumour suppression, the groups of Pandolfi, Mak, Parsons and Wu each developed transgenic mice characterized by the disruption of mouse PTEN expression on different genetic background (**Table 1.2**) using homologous recombination in mouse ES cells they have generated mutations that result in the complete recombination to delete Pten. In all models, Pten inactivation resulted in early embryonic lethality, reported at different stages of the pregnancy (E7.5, E9.5, E6.5 and E9.5 respectively). Indeed Pten –/– ES cells formed aberrant embryoid bodies and displayed an altered ability to differentiate into endodermal, ectodermal and mesodermal derivatives. Pten +/– mice and chimeric mice derived from Pten +/– ES cells showed hyperplastic-dysplastic changes in the prostate, skin and colon, which are characteristic of formation of benign tumours Di Cristofano, Pesce et al. (1998). Each group observed tumours in heterozygous mice, the onset and spectrum of tumour formation depending significantly on the genetic background but less on the type of mutation generated (Freeman, Lesche et al. 2006).

On the 129/BALB/c genetic background, the onset and spectrum of tumour development of Pten-/+ mice were significantly different from the previous reports, in almost all of which were done on the 129/C57 or 129/CD1 background (**Table 1.2**). The onset of tumour formation on the BALB/c background is significantly delayed versus other findings, which report spontaneous development of malignant tumours in <3.5 months. The spectrum of tumours is also quite different (**Table 1.2**). Previously reported

tumour incidences on the 129/C57 or 129/CD1 backgrounds were biased towards tissues and organs such as lymphoid, endometrial, adrenal, thyroid, gastrointestinal, and mammary glands. In the 129/BALB/c background, aged animals, ranging from 7 to 14 months, the incidences of lymphoid and endometrial hyperplasia or neoplasia were rather low (25% and 9%, respectively). These findings suggest that PTEN plays a critical role in cancer development, and genetic background may influence the onset, the spectrum, and the progression of tumourigenesis caused by Pten mutation (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Suzuki, Yamaguchi et al. 2001); (Freeman, Lesche et al. 2006); (Wang, Karikomi et al. 2010).

Table 1. *Pten* heterozygous tumor models: a comparison

Features	Pandolfi	Mak	Parsons	Wu
Deletion	Exons 4-5	Exons 3-5	Exon 5	Exon 5
Background	129/C57	129/CD1 or 129/C57	129/C57	129/BALB/c
Homozygotes die at:	<E7.5	E9.5	E6.5	E9.5
Tumors in heterozygotes				
Earliest detection	1.5 mo (6)	2 mo (9)	1.5 mo (7)	6 mo
Tumor types and frequencies				
Gastrointestinal hyperplasia	All? (6)	All? (9)	90% (lymphoid)	2% (inflammation)
Lymphoid hyperplasia	100% female (20) and 83% male (20)	88% (9) (T-cell lymphoma)	100% female and 45% male	33% female and 20% male
Adrenal medullary tumor	100% (21)	23% (8)	NR	None
Endometrial hyperplasia	70% (21)	80% (8)	100%	9% (45% hemangioma)
Breast	NR	49% (8)	NR	37%
Prostate	50% (21)	44% (8)	75%	90%
Thyroid	60% (21)	None (9)	30%	None

Abbreviation: NR, Not reported.

Table 1.2| A comparison between Pten heterozygous tumour models (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Suzuki, Yamaguchi et al. 2001); Freeman, (Lesche et al. 2006); (Wang, Karikomi et al. 2010)).

In addition, Pten inactivation enhanced the ability of ES cells to generate tumours in nude and syngeneic mice, due to increased anchorage-independent growth and aberrant differentiation. (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Suzuki, Yamaguchi et al. 2001); (Freeman, Lesche et al. 2006); (Wang, Karikomi et al. 2010) (Stambolic, Tsao et al. 2000). These data suggested that Pten is a tumour suppressor essential for embryonic development and Pten haploinsufficiency plays a causal role in tumourigenesis and tumour pathogenesis. Moreover the dose of Pten also affects cancer progression. A hypomorphic Pten mouse mutant series has been generated by homologous recombination with decreasing Pten activity and this study has shown that the extent of Pten inactivation dictates in a dose-dependent way prostate cancer progression, its incidence, latency, and biology affecting key downstream targets such as Akt, p27Kip1, mTOR, and FOXO3. In particular Pten hypomorphic mice (Pten hy/+), expressing 80% normal levels of Pten, develop a spectrum of tumours, with breast tumours occurring at the highest penetrance suggesting that subtle reductions in the dose of Pten expression predispose to tumourigenesis in a tissue-specific manner (Trotman, Niki et al. 2003); (Alimonti, Carracedo et al. 2010).

To further analyse the consequences of Pten loss in other organs, several tissue specific models of Pten deletion have been developed using a conditional gene-targeting approach. They mimic the effect of the loss of Pten observed in human tissues, such as brain, prostate, and breast, although not all lead to tumours.

The first three mouse models of tissue-specific inactivation of Pten were generated in 2001 (Backman, Stambolic et al. 2001); (Groszer, Erickson et al. 2001); (Kwon, Zhu et al. 2001) with the brain chosen as a target organ. PTEN is very highly frequently mutated in glioblastoma (30%, with 70% showing gene copy number reduction), the most aggressive primary brain tumour in humans (Knobbe, Merlo et al. 2002) and (2) syndromes associated with germline mutation of PTEN are characterized by neurological abnormalities. Two of these mouse models generated faithfully recapitulated the features of Lhermitte-Duclos Disease (LDD). In these mice, Pten is deleted late in the development of granule neurons of the cerebellum and

results in a cell autonomous loss of size regulation (Backman, Stambolic et al. 2001); (Kwon, Zhu et al. 2001). Indeed, several studies have established that deletion of Pten in different neuronal types during development results in marked defects in migration and patterning in brain (Backman, Stambolic et al. 2001); (Kwon, Zhu et al. 2001); (Marino, Krimpenfort et al. 2002); (Yue, Groszer et al. 2005). Although PTEN is frequently inactivated in malignant human brain tumours, PHTS is not associated with an increased incidence of brain tumours, and mice with heterozygous loss of Pten fail to develop brain tumours. Brain tumours are also not observed in conditional knockouts targeting Pten deletion in the brain (Backman, Stambolic et al. 2001); (Kwon, Zhu et al. 2001); (Marino, Krimpenfort et al. (2002); (Groszer, Erickson et al. 2001); indicating that cooperating mutations in other genes are required for the neoplastic process as demonstrated in further studies combining the loss of PTEN with simultaneous loss of p53.

Prostate cancer and glioblastoma cell lines were the first cellular models where deletion of the chromosomal region containing PTEN was reported. These findings led to the identification of PTEN as a tumour suppressor gene (Li, Yen et al. 1997); (Steck, Pershouse et al. 1997). Although human and mouse prostates are structurally dissimilar, prostate cancer progression in mice and humans is strikingly similar (Trotman, Niki et al. 2003), (Alimonti 2010 (Alimonti, Carracedo et al. 2010), (Chen, Trotman et al. 2005). Specific deletion of Pten in the prostate was achieved by crossing $Pten^{loxP/loxP}$ mice with Probasin-Cre (PB-Cre) transgenic mice. Complete loss of Pten results in the development of high-grade PIN (HG-PIN) as early as 8 weeks of age, together with the concomitant activation of cellular senescence response. HG-PIN lesions progress then to invasive prostate cancer with complete penetrance at 6 months of age. These analyses imply that loss of PTEN is critical for prostate cancer initiation (Chen, Trotman et al. 2005).

A characteristic feature of Cowden disease is the development of benign breast hamartomas that are accompanied by a higher risk of breast cancer. Although somatic PTEN mutations are detected only in a smaller fraction of breast cancer cases (Dahia 2000), LOH at the PTEN locus (10q23) is frequently found (40%) (Bose, Wang et al. 1998); The relevance of Pten in

breast tumourigenesis was initially highlighted in the mouse model of Pten germline heterozygous loss generated by Stambolic et al. Female Pten+/- developed mammary tumours at incomplete penetrance, with most of them having features of well-differentiated adenocarcinoma (Stambolic, Tsao et al. 2000). To fully understand the role of Pten in breast tumourigenesis, in 2002 Li et al. completely deleted Pten expression from mammary epithelium (Li and Thompson 2002). Females with mammary-specific Pten deletion develop tumours as early as 2 months, which had histological features similar to the basal subtypes of human breast tumours. Importantly, it has been recently shown that heterozygous inactivation of Pten leads to the formation of basal-like mammary tumours in mice, and that loss of PTEN expression is significantly associated with this subtype of breast cancer in human sporadic and BRCA1-associated hereditary breast cancers (Saal, Gruvberger-Saal et al. 2008).

In addition to the heterozygous inactivation of Pten, some groups have developed Knock In transgenic mice that express mutants of Pten with the intent of generate mice that express stable mutant Pten proteins but catalytically inactive. It is known that germline mutations in PTEN cause Cowden and Bannayan–Riley–Ruvalcaba (BRR) syndromes, two dominantly inherited disorders characterized by mental retardation, multiple hamartomas, and variable cancer risk. *In vivo* models of these pathologies were developed generating transgenic knock in mice expressing three sentinel point mutant alleles of PTEN identified in patients with Cowden syndrome: the nonsense PTEN Δ4–5 and missense PTEN C124R and PTEN G129E. PTEN Δ4–5 and missense PTEN C124R are catalytically inactive mutantsinstead, while PTEN G129E mutation causes selective loss of activity against PtdIns(3,4,5)P_3 it keeps the ability to metabolise peptide substrates. All the mutations resulted to be early embryonic lethal, mutant homozygous embryos were recovered at embryo day (E) 8.5 and E9.5, also with lower than expected frequency. In particular the development of the few mutant homozygous embryos at these ages was severely compromised, with defects in anterior-posterior patterning, failure of axial rotation, and absence of overt tissue differentiation leading to resorption by E9.5 The nonsense PTEN Δ4–5 and missense PTEN C124R

and PTEN G129E alleles, all lacking lipid phosphatase activity, cause similar developmental abnormalities but distinct tumour spectra with varying severity and age of onset. This suggested that the variable tumour phenotypes observed in patients with Cowden and BRR syndromes can be attributed to specific mutations in PTEN that alter protein function through distinct mechanisms.

Moreover, in a more recent publication, cancer-related mutations Pten C124S and Pten G129E, induce acceleration of tumourigenesis in above described Knock In mice. They specifically act by further lowering Pten activity rather than engaging alternative pathways. They inhibit the WT protein function in trans in a dominant- negative manner maybe as a result of heterodimerization. In turn, this reduced Pten lipid-phosphatase activity leads to Akt hyperactivation and increased tumourigenesis in mice. Accordingly, Pten KI Pten C124S and Pten G129E mice developed Lhermitte-Duclos disease (LDD), as previously reported in mice with total conditional Pten loss, while lesions of different histological origins not associated with Pten loss (such as sarcomas) were not observed. Thus, this study implies that patients harbouring these, or similar loss-of-function missense mutations encoding stable inactive proteins, may be more susceptible to malignant cancer and may develop it more rapidly than patients expressing reduced levels of PTEN or expressing PTEN-destabilizing mutations. PTEN mutational status may be utilized to stratify patients who may benefit from earlier and more radical therapeutic intervention modalities, potentially leading to improved prognoses. Although the possibility cannot be excluded that certain PTEN mutations may become competent toward targets not recognized by the wild-type protein in a ‘‘gain-of-function’’ scenario (Wang, Karikomi et al. 2010), these genetic analyses support a model in which the ability of mutant PTEN to interfere with WT protein function contributes to exacerbation of tumour spectrum compared to Pten heterozygosity (Papa, et al. 2014).

1.3.5 PTEN: more than a lipid phosphatase?

Most of the tumour suppressor activities of PTEN have been attributed to its ability to dephosphorylate lipid substrates and thereby regulate signalling downstream of AKT. Despite the huge potential of both the phosphatase activities of PTEN, understanding mechanisms that are independent from AKT inhibition has been an understudied area of research and it seems that the studies of PTEN's ability to metabolise PtdIns(3,4,5)P_3 and regulate AKT have dominated PTEN research for the last decade. Most of the past studies on PTEN's protein phosphatase activity have relied on the PTEN mutant G129E which selectively retains activity against peptide substrates and lacks significant PtdIns(3,4,5)P_3 phosphatase activity. Using the information from the studies on TPTE and TPIP, our group conducted a mutagenic screen to identify a PTEN mutant, which selectively lacks activity against peptide substrates. TPTE is encoded on chromosome 21 and its expression has only been identified in testis (Chen, Rossier et al. 1999). It is 551 amino acids in length and unlike PTEN possesses transmembrane domains. Activity assays imply that TPTE lacks phosphatase activity against both PtdIns(3,4,5)P_3 and phosphorylated peptide substrates. This inactivity has been attributed to the two amino acid substitutions in the phosphatase active site (+4 and +5 positions from the catalytic cysteine). Mutations of these two amino acids to those found in the PTEN phosphatase active site, reactivates TPTE making it capable of metabolizing both lipid and peptide substrates (Davidson, Maccario et al. 2010); (Leslie, Yang et al. 2007); (Walker, Downes et al. 2001). TPIP is encoded on chromosome 13, is 455 amino acids long and like TPTE, it also has trans- membrane domains. It has a slightly wider distribution compared to TPTE, but is still absent from most tissues. It seems to possesses phosphatase activity comparable to PTEN against both PtdIns(3,4,5)P_3 and PI(4.5)P_2 but lacks activity against peptide substrates (Davidson, Maccario et al. 2010); (Walker, Downes et al. 2001). The absence of PTEN's protein phosphatase activity in TPIP indicates that PTEN may have evolved specifically to retain this activity for specific functions and that PTEN's protein phosphatase activity is not just an accidental by-product of a bigger PTP active site. This strengthened the evidence for the significance of

PTEN's protein phosphatase activity and our group at the University of Dundee further investigated this in a physiological context, by performing a mutagenic screen informed by these findings to develop a 'lipid substrate only' mutant of PTENA sequence alignment of the phosphatase and C2 domains of PTEN, TPTE-reactivated (TPTE-R) and TPIP revealed 11 amino acid residues that were conserved in PTEN and TPTE-R but were different in TPIP. Single amino acid substitutions of these amino acids were made in PTEN with those found in TPIP and the activity of the mutant PTEN proteins were assayed against lipid and peptide substrates. At the 138 position, PTEN has a Tyrosine residue and the corresponding site in TPIP has a Leucine. Within the mutagenic screen, the Tyrosine at the 138 position, essential for PTEN protein phosphatase activity, was mutated to Leucine to get the PTEN Y138L mutant (**Figure 1.6**). Phosphatase assays revealed that this mutant lacked detectable activity against polyGluTyr(P) but retained activity against PtdIns(3,4,5)P_3. When expressed in PTEN null cells, PTEN Y138L was capable of reducing the levels of cellular PtdIns(3,4,5)P_3 and phospho-AKT as efficiently as wild type PTEN. Consistent with this were observed effects on downstream substrates of AKT such as GSK3 and the ability of PTEN Y138L to supress cell proliferation in a colony formation assay which was also comparable to the wild type enzyme. However, PTEN Y138L was impaired in the regulation of some processes that wild-type PTEN can inhibit. These processes include the inhibition of invasion of PTEN null U87MG cells and in the EMT of the primitive streak cells of the chick embryo indicating the requirement of PTEN's protein phosphatase activity in these processes (Davidson, Maccario et al. 2010). Although the PTEN mutant Y138L was identified in a systematic mutagenic screen, it shows the same features of a mutant found in two human cancer samples, PTEN Y138C. This mutant is characterized by the substitution of the Tyrosine at the 138 position with a Cysteine and it was found in a human endometrial carcinoma sample and a lung cancer cell line. When expressed at near physiological levels, PTEN Y138C, like PTEN wild type and Y138L reduced phospho AKT levels but had no effect in suppression of invasion of U87MG cells.

Previous studies have shown that another mutant; PTEN R308C shared the same features with PTEN Y138L (**Figure 1.6**). It is able to control cellular AKT activity but is unable to control epithelial morphogenesis and cellular invasion of U87MG cells. R308C mutation is a missense mutation characterized by the substitution of the Arginine at the 308 position with a Cysteine located in the so-called Dloop of PTEN. It was found in two different human tumour samples, a glioma and a malignant uveal melanoma It has both lipid and protein phosphatase activity and it doesn't appear to affect the stability of the PTEN protein in cultured cells.

A recent work has shown that lipid and protein phosphatase activities are both required for PTEN to regulate several biological processes such as cell migration, cellular invasion and to mediate most of its largest effects on gene expression. Through whole-genome microarray analysis, there were analyzed changes of gene expression occurring in U87MG cells grown in 3D cultures transduced with the different forms of PTEN grown. The expression of a catalytically inactive PTEN mutant had no significant effect on gene expression in these cells while Wild-type PTEN altered the expression of a large number of transcripts. PTEN G129E, which has only protein phosphatase activity, caused modest changes in the abundance of a small number of transcripts. PTEN Y138L, which has lipid phosphatase activity and can suppress cellular PIP3 levels and AKT activity, shared a large set of transcriptional responses with wild-type PTEN, but failed to affect a large group of transcripts that were the most highly responsive to the wild-type enzyme. Detailed comparisons of the transcriptional responses to wild-type and Y138L PTEN showed that almost all probes either responded similarly to both enzymes or responded only to wild-type PTEN. Very few probes were affected strongly by wild-type and weakly by Y138L, suggesting a qualitatively different signalling mechanism mediated only by wild-type PTEN, rather than a quantitative difference in the ability of these proteins to influence one pathway or set of responses. The comparison of the set of transcripts most strongly induced by wild-type PTEN, but not by PTEN Y138L, with available gene expression data from human glioblastoma samples shows significant overlap between the genes selectively induced by

wild-type PTEN and those suppressed in glioblastoma and suggest that this transcriptional control pathway may be clinically relevant (Tibarewal, Zilidis et al. 2012).

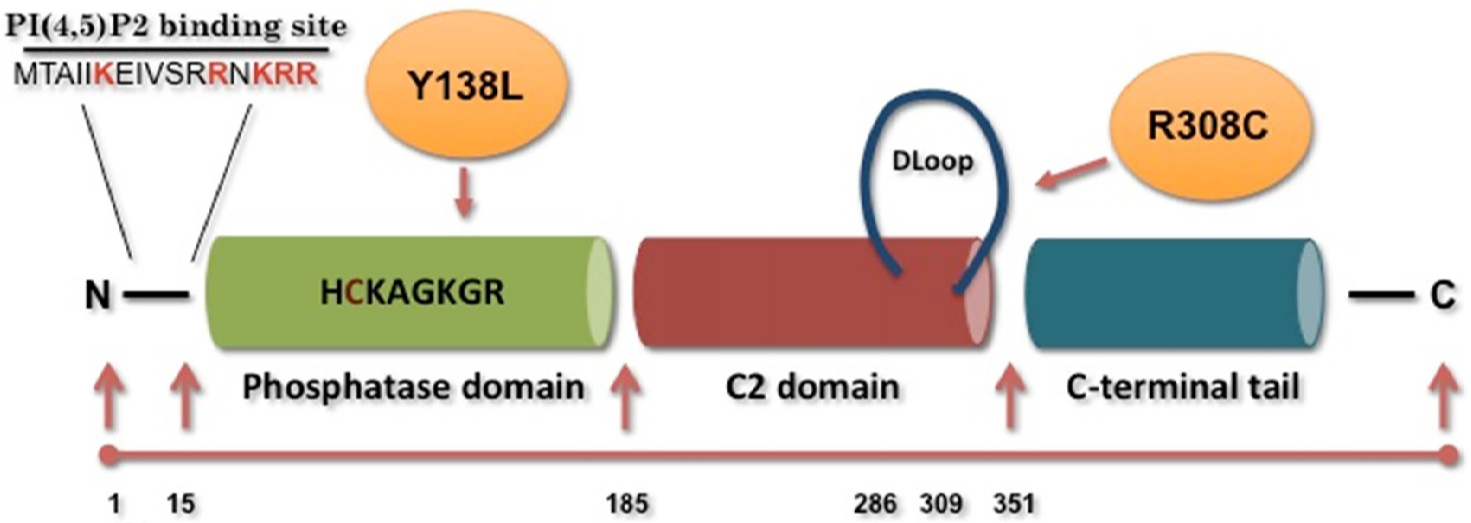

Figure 1.6| Schematic structure of PTEN indicating: the PI(4,5)P_2 binding site, the Phosphatase domain, the C2 domain, the D loop and the C-terminal tail. Red arrows show the sites of of Y138L and R308C mutations

Chapter 2

2. Materials and Methods

2.1 Animal procedure

All animal procedures were conducted following Home office guidelines under Project License authorisation (PPL 60/3989). Transgenic mice were initially generated by Taconic Artemis (Germany) and bred at the University of Dundee Transgenic Mouse Facilities.

2.1.1 Mice genotyping

Un-purified DNA was released from ear punch biopsys of mice generally taken at weaning adding 20 µl of MicroLysis Plus. The samples were placed in a Thermo Cycler used following the lysis program (Step 1: 65°C for 15 mins, Step 2: 96°C for 2 mins, Step 3: 65°C for 4 mins , Step 4: 96°C for 1 mins, Step 5: 65°C for 1 mins, Step 6: 96°C for 30 secs, Step 7: 20°C hold). The DNA was amplified in PCR using the KAPA Biosystems 2G Fast HS PCR KIT according to manufacturer's instructions, to detect the Neomicin resistance cassette inserted in the PTEN mouse gene. The products of the PCRs were detected with gel electrophoresis. Primers used in PCR are listed in Table 2.1.

Primers	
PTEN 5’ Hypomorph	5'-TGTTTTTGACCAATTAAAGTAGGCTGTG-3'
PTEN 3’ Hypomorph	5'-AAAAGTTCCCCTGCTGATGATTTGT-3'
PTEN FLOX common	5'-TTGCACAGTATCCTTTTGAAG-3'
PTEN FLOX wt	5'-GTCTCTGGTCCTTACTTCC-3'
PTEN FLOX Neo	5'-ACGAGACTAGTGAGACGTGC-3'
PTEN 5’ Y138L	5'-ATGGAAAGGAGTAAATGGATGG-3'
PTEN 3’ Y138L	5'-GGAGTAAAAGCAGGAGAATTGG-3'
PTEN 5’ R308C	5'-CACTGTAAGGTAAGTCCTATGAAGC-3'
PTEN 3’ R308C	5'-GGAGTAAAAGCAGGAGAATTGG-3'

Table 2.1| Primers for genotyping

2.1.2 Embryonic lethality determination

Pregnant female mice were culled at different stage of the pregnancy and the embryos were dissected out and snap frozen. Pictures of dissected embryos were taken when possible. The genotyping's PCR protocol was carried out on embryos as described above using DNA released from a piece of frozen tissue.

2.1.3 Tissue dissection

Mice between 38 and 42 days old were culled and the organs were immediately dissected and either snap frozen or included in formalin and fixed for Immunohistochemistry techniques. Mice identified with tumours were immediately culled according to the Home Office animal procedures. Tumours and organs were dissected and either snap frozen or incubated in in formalin and fixed for Immunohistochemistry/Chloroform.

2.1.4 RNA extraction and RT-PCR from tissues

Liver's tissue sample were reduced in small pieces and homogenised on ice using a scalpel. 1ml of Trizol (Life Technologies) was added to the homogeniser and the suspension was incubated for 5 minutes at Room Temperature. After the incubation, 100 μl of Chloroform was added to the samples mixing for 30 seconds. Samples were left at Room Temperature for 10 minutes and centrifuged for 15 minutes at 4ºC. For each liver tissue sample RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. RNA was eluted from columns in nuclease-free water. 500 ng of RNA was run on a 2% agarose gel to verify the presence of two bands (28S and 18S rRNA) at approximately 4.5 and 1.9 kb, and RNA integrity.

Random hexamer-primed cDNA was synthesised using Sprint RT-random hexamer kit (Clontech) following the manufacturer's protocol. Full length

PTEN cDNA was then amplified by PCR with the primer pair listed below (**Table 2.2**). The products were sequenced by Sequencing Service, University of Dundee, UK, using internal primers (**Table 2.2**).

Primers	
PTEN 5’ UTR mouse F	5'-CATGTTGCAGCAATTCACTG-3'
PTEN 3’ UTR mouse R	5'-GGTATTTTATCCCTCTTGATAAG-3'
GAPDH mouse F	5’-CAACTCCCACTCTTCCACCTTCG-3’
GAPDH mouse R	5’-GTAGGGAGGGCTCAGTGTTGGG-3’

Table 2.2| Primers for amplifying cDNA for sequencing

2.2 In vitro experiments

2.2.1 In vitro materials

Suppliers for main reagents are listed in Table 2.3.

Reagent	**Suppliers**
1 Kb Plus Ladder	Invitrogen
Agarose	Invitrogen
Albumin Essentially fatty acid free	Sigma
Carbenicillin	Sigma Aldrich
Benzamidine	Sigma Aldrich
Bradford reagent	Sigma
BSA Bovine Serum Albumin Fraction V	Roche
Chemiluminiscent reagent	Millipore
DTT	Sigma
HRP-linked secondary antibodies	Pierce Biotechnology Inc.
Kanamycin	Melford
Kod Hot Start Polymerase	Novagen

LDS Sample Buffer	Invitrogen
Luria Broth-LB (powder)	Sigma
MicroLysis Plus	Web Scientific
NuPAGE Novex systems Bis-Tris gels	Invitrogen
PMSF	Sigma
Primary antibodies	See Table x
Primers	MWG
Promega Dual Luciferase Reagents Kit	Promega
PVDF membrane	Perkin elmer
QIAshreader columns	Qiagen
Qiaprep Miniprep Kit	Qiagen
Qiaprep Maxiprep Kit	Qiagen
See Blue Plus2 Prestained standard	Invitrogen

Table 2.3| *In vitro* reagents

Plasmid expression vectors for PTEN wild-type, PTEN C124S, PTEN Y138L, were already in use in the lab. Luciferase promoter reporter plasmids used for luciferase assays were kindly provided by other laboratories (**Table 2.3**).

2.2.2 Preparation of agarose gel

Agarose gels were prepared by dissolving agarose in TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA) by heating. 10 µl of SYBR safe DNA gel stain (Invitrogen) was added and diluited in 100ml of liquid agarose and the whole solution agarose poured into gel plates containing well combs. When the gel was solidified, it was submerged by adding TAE buffer and the combs were removed.

DNA samples mixed with loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (w/v) glycerol) were loaded into individual wells.

The gel was run at 100 V for 1 hour until the blue tracking dye had migrated about 75% of the distance to the end of the gel. The DNA bands were then visualised with U.V. light. 1Kb Plus DNA Ladder (Invitrogen) was used as marker.

2.2.3 Preparation of chemically competent Escherichia coli cells

Chemically competent *E. coli* cells of the strains TOP10 (DNA manipulation), and BL21 (protein purification) were prepared using the treatment based on the RbCl method. 5 ml of LB medium was inoculated with E.coli and left to grow overnight at 37ºC with constant shaking. The overnight culture was then diluted into 500 ml of LB medium and left to grow at 37ºC for a time required to obtain an OD of 0.5 at 550 nm. The culture was chilled on ice for 5 minutes and then harvested by centrifugation at 4000 rpm (1600xg) for 10 minutes at 4ºC. The pellet was resuspended in 40 ml of 30 mM KOAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, and 15% glycerol (pH 5.8). Cells were harvested as above and resuspended in 7.5 ml of 10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, and 15% glycerol (pH 6.5). Aliquots of desired volume were prepared and snap frozen in a dry ice/methanol bath and stored at -80ºC.

2.2.4 Transformation of Escherichia coli with plasmids

Chemically competent *E. coli* cells of the strains TOP10 (DNA manipulation), BL21 (protein purification), prepared as described in Section 2.2.2, were used for transformations. 100 ng of DNA (the amount can vary depending on the transformation efficiency of the vector used) was incubated with 10 µl of E. coli competent cells for 20 minutes on ice. The cells were, successively, heated at 42ºC for 45 seconds and then placed on ice and left to cool for two minutes, before the addition of 250 µl of SOC medium (Super Optimal Broth with glucose). The cells were then incubated for 1 hour at 37ºC with constant shaking. The bacteria were then plated on selective LB agar plates containing the appropriate antibiotic (50 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated at 37ºC overnight.

2.2.5 Purification of plasmid DNA

Large amount of DNA is generally required for cell transfection. Therefore, a small scale (mini-prep) or large scale (maxi-prep) plasmid DNA preparation was performed. 4 ml of LB medium containing the appropriate antibiotic was inoculated with a single colony picked from the selective LB agar plates, and incubated at 37 ºC overnight with constant shaking. After 24 hours, the bacterial culture was pelleted at 3250 rpm (1500xg) for 15 minutes and the plasmid DNA purified using a 96-well robot (Dundee University service) or a Mini-prep kit, purchased from Qiagen (QIAprep Miniprep Kit), according to the manufacturer's instructions.

For a large scale DNA purification, the starter culture was diluted into 500 ml of selective LB medium and incubated at 37 ºC overnight with vigorous shaking. Cells were then harvested by centrifugation and the plasmid DNA purified using the QIAfilter Plasmid Maxi Kit protocol (Qiagen).

DNA concentration was determined by measuring the absorbance of a dilution of the DNA in water at OD260. The OD260 value was multiplied by

the dilution factor and using the relationship that an A260 of 1.0 is 50 μg/ml of pure DNA.

2.3 Proteins analysis

2.3.1 Preparation of cell and tissues lysates

The preparation of cell lysate was carried out on ice. The culture medium was removed and the cells were washed in ice-cold PBS twice. Lysis buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 1mM EGTA, 1mM EDTA, 5mM sodium pyrophosphate, 10mM β-glycerophosphate, 50mM sodium fluoride, 0.2mM PMSF, 1mM benzamidine, 1mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 270mM Sucrose) was added and the cells were left on ice for 30 minutes prior to being scraped off. Cells were collected and centrifuged at 14000 rpm (6000xg) for 20 minutes at 4ºC to separate the total soluble protein (supernatant) from the insoluble cellular debris (pellet).

The initial preparation of tissues lysate was carried out on dry ice. Frozen tissues were reduced into small pieces using a deep liquid nitrogen scalpel and homogenised in cold Lysis Buffer (25mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP-40, 1mM EGTA, 1mM EDTA, 5mM sodium pyrophosphate, 10mM b-glycerophosphate, 50mM sodium fluoride, 0.2mM PMSF, 1mM benzamidine, 1mM sodium orthovanadate, 0.1% 2-mercaptoethanol, 270mM Sucrose, 1mM Microcystein) in a homogeniser. Suspensions were centrifuged at 14000 rpm (6000xg) for 20 minutes at 4ºC to separate the total soluble protein (supernatant) from the cellular debris (pellet).

The supernatants were then transferred to new tubes and the protein concentration estimated as described below. Samples for western blotting analysis were then made as described below.

2.3.2 Estimation of protein concentration

The concentration of protein samples was determined using the common Bradford method and by reference to absorbance measurements obtained for a series of standard BSA (Bovine Serum Albumin) dilutions. 10 µl of sample was mixed with 200 µl of Bradford reagent (Sigma) in a 96 well plate. After shaking the plate for 30 seconds and 3 minutes of incubation at room temperature, the absorbance was measured at 595nm with a plate reader. The measurement obtained for the blank was subtracted from all other samples measurements (BSA and purified protein) and a standard curve was prepared by plotting the standard concentration (µg/ml) vs. measurement of the protein.

2.3.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was used to separate proteins of interest, by size, shape and charge and was performed using NuPAGE Novex system Bis-Tris gels, either 4-12% gradient or 10% (Invitrogen). The gels were loaded using denaturing and reducing conditions following the manufacturer's instructions and run for 60 minutes at 200 V in the presence of MOPS running buffer (20X stock: 1 M MOPS, 1 M Tris base, 69.3 mM SDS, 20.5 mM EDTA free acid).

2.3.4 Preparation of samples

The desired amount of purified protein or protein from cell and tissue lysates was mixed with 1X LDS sample buffer (Invitrogen) and incubated at 70ºC for 10 minutes.

To run the samples under reducing conditions, β-mercaptoethanol was added to the sample buffer. The samples were then mixed and loaded on the gel side by side with molecular weight marker SeeBlue Plus2 Prestained standard (Invitrogen).

2.3.5 Immunological protein detection by Western Blotting

After electrophoresis, proteins were transferred from a gel to a wetted PVDF membrane (Perkin Elmer), for 1 hour at 30 V, in the presence of transfer buffer (500 mM Bicine, 500 mM Bis-Tris, 20.5 mM EDTA) using a tank wet transfer system. Following transfer, the PVDF membrane was dried for 30 minutes and then blocked with a solution of 5% (w/v) low fat milk protein or 5% (w/v) BSA made up in TBS-T (200 mM Tris-HCl pH 7.6, 137 mM NaCl, 1% (v/v) Tween 20).

The blocked PVDF membrane was washed over 30 minutes with three buffer changes of TBS-T, before its incubation at 4ºC with a primary antibody made up in 5% (w/v) low fat milk protein or 5% (w/v) BSA (**Table 2.4**). The time of incubation is relative to the antibody used. The primary antibody was washed off over 30 minutes with three buffer changes of TBS-T, followed by incubation with the appropriate HRP-linked secondary antibodies (Pierce Biotechnology Inc) made up in 5% (w/v) low fat milk, for 1 hour at room temperature. The unbound antibody was then removed by washing the membrane over 30 minutes with three buffer changes of TBS-T. Proteins were then detected using chemiluminescence reagents (Millipore). The two solutions were mixed and poured on the membrane according to the manufacturer' protocol. The image was acquired directly using a chemiluminescence-compatible digital imaging system (CCD camera from GE Healthcare), which was also used used for detection.

2.3.6 Western blot reprobing

PVDF membrane can be sequentially incubated with different antibodies after using the stripping procedure by which the first antibody is removed from the blot.

After washing off the chemiluminescence reagent with multiple TBS-T washes, the blot was incubated in a stripping solution (100 mM β-

mercaptoethanol, 2% (w/v) SDS, 50 mM Tris-HCl pH 6.8) for 30 minutes at 50ºC with agitation. The blot was then washed for over 30 minutes with three buffer changes of TBS-T, followed by the blocking step for the next immunodetection.

Antibody	Species	Supplier
Primary antibodies		
AKT	Rabbit polyclonal	Cell Signalling
p-AKT (S473)	Rabbit polyclonal	Cell Signalling
p-AKT (T308)	Rabbit polyclonal	Cell Signalling
FOXO-1	Sheep polyclonal	Cell Signalling
p-FOXO1	Rabbit polyclonal	Cell Signalling
GAPDH	Mouse monoclonal	Millipore
p-GSK3 alpha/beta (S21/9)	Rabbit polyclonal	Cell Signalling
GSK3-3alpha/beta	Rabbit polyclonal	Cell Signalling
mTOR	Rabbit polyclonal	Cell Signalling
p-mTOR (Ser2448)	Rabbit polyclonal	Cell Signalling
p70 S6 kinase (T389)	Rabbit polyclonal	Cell Signalling

p-p70 S6 kinase (T421/S424)	Rabbit polyclonal	Cell Signalling
PRAS40	Rabbit polyclonal	Cell Signalling
p-PRAS40 (p-T246)	Rabbit polyclonal	Cell Signalling
PTEN (17A)	Mouse monoclonal	AbCAM
PTEN (6H2.1)	Rabbit polyclonal	Alexis
Vinculin	Rabbit polyclonal	Cell Signalling
Secondary antibodies		
Anti-mouse	Rabbit	Pierce
Anti-rabbit	Goat	Pierce
Anti-sheep	Rabbit	Pierce

Table 2.4| List of antibodies

2.4 Cell culture and cell-based assays

2.4.1 2D culture of cells

U87MG and DBTRG-05MG glioblastoma cells were originally purchased from the ECACC; Human embryonic kidney 293T cells were originally provided by Michelle West and Colin Watts (Division of Cell Signalling and Immunology, University of Dundee).

Human embryonic kidney 293T cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1 % L-Glutamine. U87MG cells were grown in DMEM supplemented with 10% FBS, 1 % L-Glutamine, 1% NEAA, 1% Sodium Pyruvate. DBTRG cells were grown in RPMI supplemented with 10% FBS, 1% NEAA, 1% Sodium Pyruvate. All the cell lines were incubated at 37°C in 5% CO_2/95% air.

For passaging of cells, the monolayer was washed once with warm PBS and then incubated with dissociation buffer (Life technologies) at 37°C until the cells detached from the surface. The cells were suspended in serum containing media to inactivate the buffer and harvested by centrifugation at 150xg for 5 minutes. The cells were then seeded into flasks at 1:5 in complete medium. The media was changed routinely and the cells were split again once they were confluent. Cells were counted using a haemocytometer. Normal tissue culture plasticware was used for all other cells. For cryopreservation of cells, cells were trypsinised, harvested and resuspended in freezing medium containing 90% FBS and 10% DMSO at 3x106 cells/ml. 1ml aliquots were made in cryovials. The vials were frozen slowly using a controlled rate cell freezer before they were stored under liquid nitrogen.

2.4.2 3D culture of cells

3D culture of cells was performed using U87MG cells to analyse the luciferase activity of the promoter repoter plasmids transfected. U87MG cells

were transfected using TransIT, procedure described in the specific section. Growth Factor Reduced Matrigel (BD Biosciences) was defrosted overnight on ice in the cold room. 3D cultures were set 18hrs after transfection. 150µl of defrosted matrigel was added to pre-chilled 24wells plates placed on ice in 6 well plates. All matrigel dispensing used pre-chilled pipette tips. The matrigel was allowed to solidify at 37°C for 30 minutes. Cells were trypsinised and counted. 50,000 cells were plated on top of the solidified matrigel and allowed to attach for 30 minutes at 37°C. 100µl of matrigel was then poured over the layer of cells to form a sandwich of cells between two layers of matrigel. The matrigel was allowed to solidify for 30 minutes at 37°C. 500µl of complete U87MG media was added to the wells and the cells were incubated in a tissue culture incubator for 18hrs and allowed to invade through the 3D matrix. After incubation, the cultures were washed once with PBS and lysated with Passive Lysis Buffer (Promega). The dual luciferase assay was then performed, whom procedure is described below in details.

2.4.3 Cell transfection

DNA was introduced into cells using non-viral methods (transfection). Different methods were used depending on the efficiency of transfer and cell types.

2.4.4 Calcium phosphate transfection

Calcium phosphate transfection method has been used to transfect HEK293T cells. 2 hours prior to transfection, cells were split and plated at 30% confluence in 10 cm dishes containing 10 ml of medium.

DNA (10 µg) was added to 61 µl of 2 M calcium chloride ($CaCl_2$), which was then made up to a volume of 500 µl. This mix was slowly added to 500 µl of 2x HEPES buffered solution (HBS, 50 mM HEPES pH 6.95, 280 mM sodium chloride, 15 mM disodium hydrogen phosphate). The CaCl2/HBS/DNA precipitate was directly added to the cells, covering as much of the plate as

possible. After 24 hours of incubation, the cells were changed into fresh medium and then incubated for a further 24 hours.

2.4.5 TransIT-LT1 transfection

$5x10^5$ cells were plated in 24 wells plates containing medium, approximately 12 hours before transfection. Immediately before transfection, serum-free Opti-MEM (31.25 µl) was mixed with warm TransIT-LT1 reagent (3 µl of TransIT-LT1: 1 µg of DNA) (Mirus, Madison, WI, USA). The required amount of plasmid DNA was gently added to the diluted TransIT-LT1 reagent. The mix was incubated at room temperature for 15-30 minutes before being added dropwise to the cells. The plates were gently agitated to distribute the TransIT-LT1 reagent-DNA complexes. After 24 hours of incubation, the cells were changed into fresh medium and then incubated for a further 24 hours.

2.4.6 Dual-Luciferase assay

For inhibitor treatments, 1µM GDC0941 (PI3K inhibitor) was added to the culture medium 24 hours before performing the assay. To assay luciferase activity in cells transfected with luciferase expression vectors, co-transfected cells were washed in 1X PBS at Room Temperature and 100 µl (2D culture) or 400 µl (3D culture) of Passive Lysis Buffer (Promega) was added to each well. After 15 minutes at Room Temperature lightly shaking, 20 µl of the lysates was transferred to a 96 wells white plate. The luciferase assay was performed using the dual-injector, plate-reading luminometer **(**Omega Star, BMG Labtech) and the Promega Dual Luciferase Reagent Kit accordingly to manifacturer's protocol. The Dual-Luciferase® Reporter (DLR™) Assay System provides an efficient means of performing two reporter assays. In the DLR™ Assay, the activities of firefly (Photinus pyralis) and Renilla (Renilla reniformis or sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a luminescent signal lasting at least

one minute. After quantifying the firefly luminescence, this reaction is quenched and the Renilla luciferase reaction is initiated simultaneously by adding Stop & Glo® Reagent to the same sample. Both assays can be completed in about 4 seconds using a luminometer with reagent auto-injectors. In the DLR™ Assay System, both reporters yield linear assays with attomole ($<10^{-18}$) sensitivities and no endogenous activity in the experimental host cells. Furthermore, the integrated format of the DLR™ Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

Construct	Number of constructs	Reference
HGMR1	1	Lee HJ et al. 2012
GADD45	1	Takahashi S et al. 2001
GDNF	1	Brodbeck S et al. 2004
WISP-2/CCNF	2	Fritah A et al. 2006
IGFBP1	2	Sutherland Lab, University of Dundee
FOXO1	1	Essaghir A et al. 2009
LKB1	3	Lützner N et al. 2012
ILRB2	2	Verma VK et al. 2012
MZF1 p55	1	Deng Y et al. 2013
β-Catenin	3	Liu G et al. 2012
FHRE	1	Addgene 1789
GADD45	1	Addgene 8358
ILR2	1	Addgene 10959
ERE	1	Addgene 11354
β-Catenin	1	Addgene 12456

Axin2	1	Addgene 21275
VEGFR2	1	Addgene 21306/21307
STAT	1	Addgene 37392

Table 2.5| Luciferase promoter reporter plasmids used in the dual-luciferase assay

2.5 Buffers and solutions

Buffer	Composition
DNA Manipulation	
TAE buffer	40mM Tris□HCl, pH 8.0
	20mM acetic acid
	1mM EDTA
DNA elution buffer	50mM Tris□HCl, pH 8.0
6X DNA Loading dye	0.25% bromophenol blue
	0.25% xylene cyanol FF
	30% glycerol in water
Protein purification	
Tissue Lysis Buffer	25mM Tris□HCl pH 7.4
	150mM NaCl
	1% NP□40
	1mM EGTA
	1mM EDTA
	5mM sodium pyrophosphate

	10mM β -glycerophosphate
	50mM sodium fluoride
	0.2mM PMSF
	1mM benzamidine
	1mM sodium orthovanadate
	0.1% 2□mercaptoethanol
	270mM Sucrose
	1mM Microcystein
SDS PAGE, Western blotting and agarose gels	
MOPS running buffer	50mM MOPS
	50mM Tris Base
	0.1% SDS
	1mM EDTA, pH 7.7
Transfer Buffer	25mM Bicine
	25mM Bis Tris
	1mM EDTA
TBS-T 10X	0.2 M Tris base pH

	1.37M NaCl
	1% Tween
Stripping buffer	0.2M Glycine pH 2.2
	0.1% SDS
Cell culture and cell based experiments	
PBS	137mM NaCl
	2.7mM KCl
	4.3mM Na_2HPO_4
	1.47mM KH_2PO_4
	pH 7.4
Cell Lysis Buffer	25mM Tris□HCl pH 7.4
	150mM NaCl
	1% NP□40
	1mM EGTA
	1mM EDTA
	5mM sodium pyrophosphate
	10mM β □glycerophosphate

	50mM sodium fluoride
	0.2mM PMSF
	1mM benzamidine
	1mM sodium orthovanadate
	0.1% 2□mercaptoethanol
	270mM Sucrose

Table 2.6| Buffer and solutions.

Aim

PTEN is a tumour suppressor gene that is found mutated in many human cancers. Most of its tumour suppressor activities and effects on gene expression appear related to its ability to inhibit the PI3- Kinase/AKT pathway and therefore suppress cell proliferation, survival and migration. Recent studies in our lab have shown that two mutants, PTEN Y138L and PTEN R308C, have the ability to control AKT activity but not cell migration and invasion.

The aim of this project is to further understand how PTEN regulates tumourigenesis. To do this, two transgenic knock in mice lines were developed expressing PTEN Y138L and PTEN R308C mutants. The aim of this work was to analyse the potential of these two mutants to lead to tumour formation and to influence embryo viability.

In parallel, I have mined gene expression profiling data to investigate how PTEN and PTEN Y138L regulate gene expression. Luciferase promoter reporter assays were used to study genes whose transcription is regulated by PTEN. The goal of this latter work is to provide tools to easily reveal AKT-dependent and AKT-independent transcriptional responses controlled by PTEN to investigate mechanisms by which PTEN controls cellular phenotype.

Chapter 3

3. Results and Discussions

3.1 Phenotypic analysis of Pten Y138L and R308C Knock In transgenic mice

Mouse models of human cancer have played a vital role in understanding tumorigenesis and answering experimental questions, like interactions between cancer and normal cells in vivo, that other systems cannot address. Advances continue to be made that allow better understanding of the mechanisms of tumour development, and therefore the identification of better therapeutic and diagnostic strategies. Many research groups have used mice models to reconstruct in vivo the effects of PTEN loss and mutations on tumour development and cancer progression. These studies confirm that PTEN plays a critical role in cancer development, and genetic background may influence the onset, the spectrum, and the progression of tumorigenesis caused by Pten mutation (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Suzuki, Yamaguchi et al. 2001); (Freeman, Lesche et al. 2006); (Wang, Karikomi et al. 2010).

Most of the tumour suppressor activities of PTEN have been mainly attributed to its ability to dephosphorylate lipid substrates and thereby regulate signalling downstream of AKT. Despite the huge potential of both the phosphatase activities of PTEN, either by regulating PI3K effecting AKT or other pathways, such as RAC1, and by dephosphorylating heterolougous substrates, like FAK and SRC, understanding mechanisms that are independent from AKT inhibition has been an understudied area of research and it seems that the studies of PTEN's ability to metabolise PtdIns(3,4,5)P_3 and regulate AKT have dominated PTEN research for the last decade.

A recent work published in our lab has shown that lipid and protein phosphatase activities are both required for PTEN to regulate several biological processes such as cell migration, cellular invasion and to mediate most of its largest effects on gene expression. Through whole-genome microarray analysis, there were analyzed changes of gene expression occurring in U87MG cells grown in 3D cultures transduced with the different forms of PTEN grown (Tibarewal, Zilidis et al. 2012). Using the information from the studies on TPTE and TPIP, our group conducted a mutagenic screen to identify a PTEN mutant, which selectively lacks activity against peptide substrates. Within the mutagenic screen, the Tyrosine at the 138 position, was mutated to Leucine to get the PTEN Y138L mutant. Phosphatase assays revealed that this mutant lacked detectable activity against polyGluTyr(P) but retained activity against PtdIns(3,4,5)P_3. When expressed in PTEN null cells, PTEN Y138L was capable of reducing the levels of cellular PtdIns(3,4,5)P_3 and phospho-AKT as efficiently as wild type PTEN. Consistent with this were observed effects on downstream substrates of AKT including GSK3 and the ability of PTEN Y138L to supress cell proliferation in a soft-agar colony formation assay that was also similar to the wild type enzyme. However, PTEN Y138L was impaired in the regulation of some processes that wild-type PTEN can inhibit, including invasion and control of epithelial morphology.

Although the PTEN mutant Y138L was identified in a systematic mutagenic screen, it shows the same features of a mutant found in two human cancer samples, PTEN Y138C. This mutant is characterized by the substitution of the Tyrosine at the 138 position with a Cysteine and it was found in a human endometrial carcinoma sample and a lung cancer cell line – NCIH96 cells. Previous studies in the lab have shown that another mutant, PTEN R308C shared the same features with PTEN Y138L and Y138C. It is able to control AKT activity and cell migration in vitro but is unable to control epithelial morphogenesis and cellular invasion of glioma cells, DBTRG and U373 cell lines. PTEN R308C is a missense mutation characterized by the substitution of the Arginine at the 308 position with a Cysteine located in the so-called Dloop of PTEN. It was found in two different human tumour samples, a

glioma and a malignant uveal melanoma. PTEN R308C has both lipid and protein phosphatase activity and displays stability to wild-type PTEN in cultured U87MG cells.

Having established in cultured cells that these two cancer-related mutations show partial loss of function compared to WT PTEN protein, this lead our lab to generate two Knock In transgenic mouse lines that express PTEN Y138L and PTEN R308C respectively. The reason to develop the two transgenic mouse lines was to further analyse how PTEN could regulate tumorigenesis *in vivo* and furthermore, if present, understand those pro-cancer mechanisms that are independent from AKT inhibition.

3.1.1 Generation of Knock In Pten Y138L and Pten R308C Transgenic mice

The two new transgenic mouse lines, Pten KI Y138L and Pten KI R308C were generated as a service by Taconic Artemis. They are constitutive Knock-Ins of the point mutations (KI-PM) Pten Y138L and Pten R308C and the strategy used for the generation of them is described below. The targeting vectors were generated using BAC clones from the C57BL/6J RPCIB-731 BAC library and transfected into the Taconic Artemis C57BL/6N Tac ES cell line. Through homologous recombination Y138L and R308C mutations were introduced into exon 5 and exon 8 of the Pten gene respectively, together with the positive selection marker (Puromycin resistance - PuroR) that was flanked by FRT sites and was inserted into intron 4 and intron 8 respectively. Homologous recombinant clones were then isolated using positive (PuroR) and negative (Thymidine kinase - Tk) selection. The constitutive KI-PM alleles were obtained after Flp-mediated removal of the selection marker. These KI-PM alleles express the mutated Pten Y138L and Pten R308C proteins, in particular the only difference from the native allele is each mutation and a single FRT site in the neighboring intron (**Figure 3.1**).

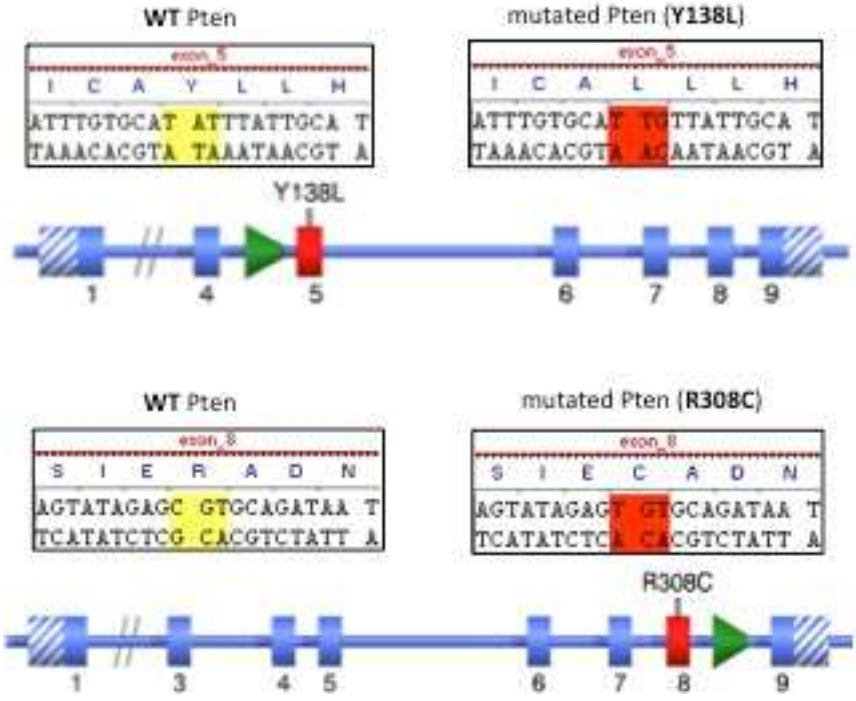

Figure 3.1| Sites of insertion of the two mutations for each transgenic line developed as a service by Taconic Artemis. a. The Y138L (AT>TG) mutation was introduced into exon 5 of Pten gene. b. The R308C (C>T) mutation was introduced into exon 8 of Pten gene. Green arrows correspond to FRT regions and the red boxes represent the two mutant exons, for Pten Y138L and Pten R308C respectively.

3.1.2 KI Y138L and KI R308C mice genotyping and PTEN gene sequencing

In performing the in vivo characterization of these new Pten KI mice, I initially aimed to investigate two critical aspects: the presence of Pten Y138L and Pten R308C mutations in the correct sites and their effects on Pten protein stability.

First, I wanted to be sure that any phenotypical change that I would have seen in the transgenic mice was due only to the presence of the mutations of our interest. First, to screen the mouse genotypes I have extracted DNA from all the transgenic mice in the breeding, from ear punches. I have then amplified the gene and performed a PCR in order to get the genotype of the mice. Both the KI Pten mutant are about 50 base pairs (bp) more than Pten WT gene because of the presence of two flanking regions of the PuroR and FRT. The PuroR site was removed after being used during the generation of the mice as selection marker. The difference in dimension of the PCR products allowed me to distinguish if the mouse was expressing two alleles of WT Pten (homozygous mouse, one single band of 250bp), one allele of WT Pten and one of KI Pten mutated gene (heterozygous mouse, two bands at 300 bp and 200 bp) or two alleles of KI Pten mutated gene (homozygous mouse, one single band of 300bp). To confirm that this PCR product size-based genotyping assay reflected the presence of the relevant point mutation, I directly sequenced the expressed mRNA of Pten from three mouse liver samples.

This also allowed me to check that there were no other mutations within the Pten gene other than our KI mutations of interest that could influence the phenotype of the mice. RNA was isolated from the liver of KI Pten Y138L and KI Pten R308C mice and, after amplifying the Pten gene through PCR I have sequenced the gene using the Sequencing Service of the University of Dundee (**Figure 3.2**).

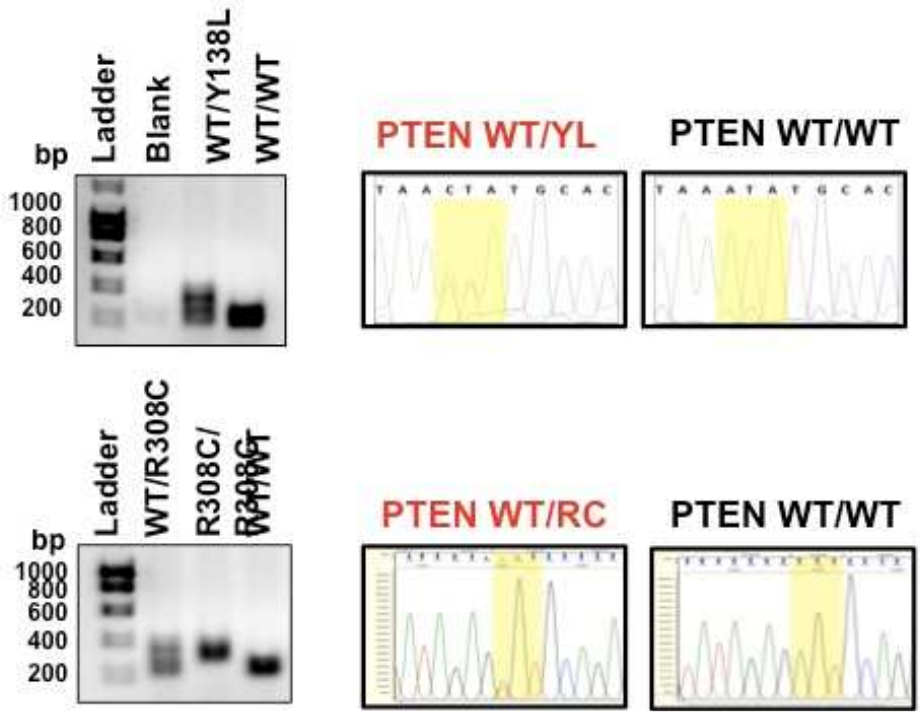

Figure 3.2| Genotyping gels of Pten gene amplified from DNA samples released from transgenic mice ears biopses for KI Y138L(a) and KI R308C (b) and sequencing of Pten -exon5 for Y138L (a) mutation and Pten -exon8 for R308C(b) mutation amplified from cDNA of respective mice liver tissues. Electropherograms show expression of both Pten wt and Pten mutated alleles

From the sequencing of the Pten gene from three mice per line, I could verify that for both KI Pten Y138L and KI Pten R308C only the missense mutation inserted during the generation of the mice was present. This result gave me confidence that all the analysis and experiments conducted with the new cancer mouse models, both in vivo and ex vivo, would allow me to further understand how Pten Y138L and Pten R308C themselves could influence and regulate tumorigenesis in vivo.

Also, since many PTEN missense point mutations render PTEN unstable (Lee, Yang et al. 1999), I measured the mutant Pten proteins levels in our Pten KI mice. I decided to analyze healthy tissues from aged matched mice (3 males and 3 females from 38 to 42 days old) from the 6 transgenic mice groups in our breeding. Brain tissues samples were dissected, snap frozen and later homogenized in lysis buffer. Pten expression was analyzed in Western Blot for 3 mice per group (**Figure 3.3**).

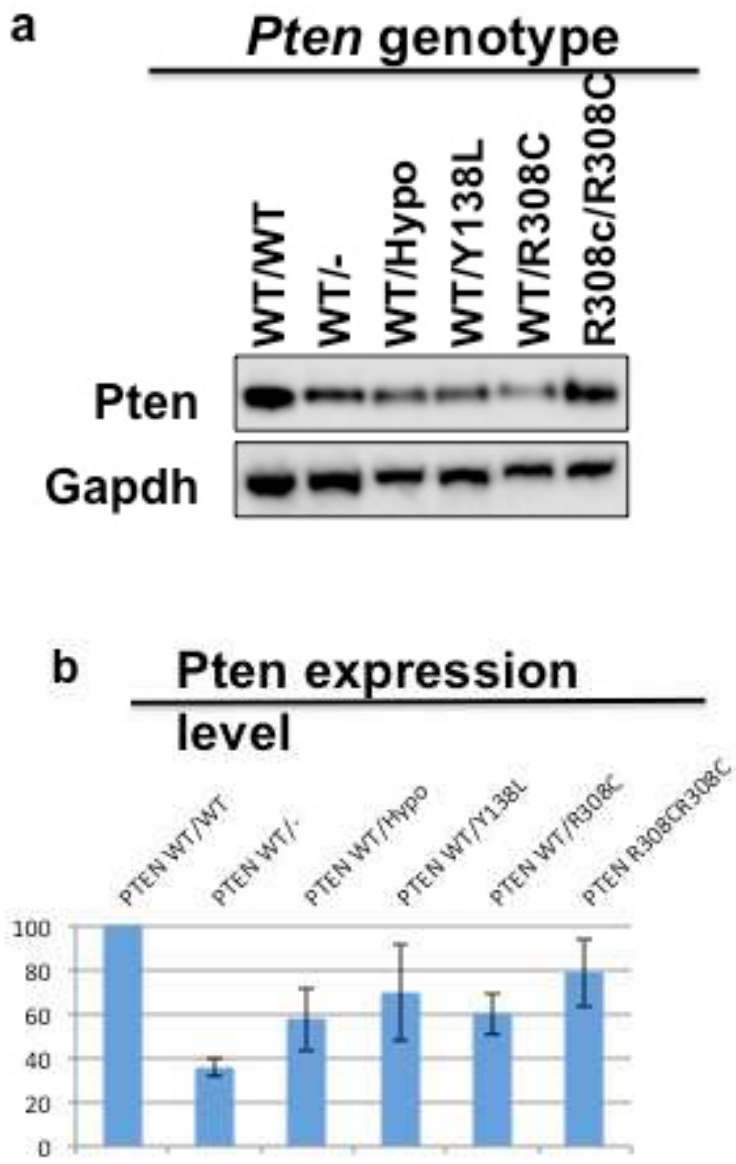

Figure 3.3| a. Healthy brain tissues were dissected post-mortem from Pten WT (WT/WT), Pten Null (-/WT), Pten Hypomorph (Hypo/WT), KI Pten Y138L (YL/WT) and KI Pten R308C het (RC/WT) and hom mice (RC/RC). Pten expression was investigated by western blotting of tissues lysates using specific antibodies. **b.** Percentage of Pten expression normalized on Gapdh in mice brain tissues samples analysed in WB related to Pten WT expressing mice. Error bars show STD, n=3.

In the figure is shown one representative experiment out of 3, while the quantification data are related to 3 independent experiments of tissue samples from 3 mice for each group. These results show that both the KI Pten mutants are expressed in healthy tissues even though with a less amount of protein compared to WT Pten. The quantification analyses revealed that Pten R308C is expressed in heterozygosis, about 70-80%, and homozygosis, about 95%, when compared to WT Pten expressing mice, set as 100%. Pten Y138L may be expressed in heterozygosis, about 70% compared to Pten WT expressing mice, but, without the presence of samples from mice carrying the Y138L mutation in homozygousis, I would need other analysis in order to confirm the expression of the Pten Y138L mutant in het mice. For this reason, there were generated by Dr. Priyanka Tibarewal Mouse Embryonic Fibroblast (MEFs) cell lines from embryos of different genotypes, including Pten YL/WT and YL/YL. Experiments carried in MEFs will be described later in the thesis. The reduced expression of Pten Y138L and Pten R308C, compared to the WT protein, could be related to less stability of the mutants that could lead to a decreased expression of the proteins.

3.1.3 Pten Y138L and Pten R308C analysis of influence in embryos viability

To examine the role of PTEN in ontogenesis and tumour suppression, the groups of Pandolfi, Mak, Parsons and Wu each developed transgenic mice characterized by the disruption of mouse Pten expression on different genetic background. (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Suzuki, Yamaguchi et al. 2001); (Freeman, Lesche et al. 2006); (Wang, Karikomi et al. 2010). In all models, Pten inactivation resulted in early embryonic lethality, at different stage of the pregnancy (E7.5, E9.5, E6.5 and E9.5 respectively). Indeed Pten –/– ES cells formed aberrant embryoid bodies and displayed an altered ability to differentiate into endodermal, ectodermal and mesodermal derivatives (Di Cristofano, Pesce et al. 1998). Futhermore *in vivo* models of Cowden and Bannayan–Riley–Ruvalcaba (BRR) syndromes were developed generating transgenic Knock In

mice expressing three sentinel alleles representing PTEN mutants identified in patients with Cowden syndrome: the nonsense PTEN Δ4–5 and missense PTEN C124R and PTEN G129E. PTEN Δ4–5 is a very unstable protein fragment whose expression cannot even be detected and is catalytically inactive. The missense PTEN C124R is a catalytically inactive mutant, instead, while PTEN G129E mutation causes selective loss of activity against PtdIns(3,4,5)P_3 it is stable and keeps the ability to metabolise peptide substrates. All the mutations resulted in early embryonic lethality and mutant homozygous embryos were recovered at embryo day (E) 8.5 and E9.5, also with lower than expected frequency. In particular the development of the few mutant homozygous embryos was severely compromised, with defects in anterior-posterior patterning, failure of axial rotation, and absence of overt tissue differentiation leading to resorption by E9.5 Wang, Karikomi et al. (2010).

Previous transgenic mouse model lacking Pten or expressing or PTEN mutants resulted to be embryonic lethal (Di Cristofano, Pesce et al. 1998) (Trotman, Niki et al. 2003); (Wang, Karikomi et al. 2010). Also, mice expressing reduced [hypomorphic] level of Pten affected homozygous embryos viability, causing a reduction in the numbers of expected homozygous mice born (Trotman, Niki et al. 2003). Ever since these findings, I first evaluated the effect of Pten Y138L and Pten R308C on embryonic development by examining offspring derived from intercrosses between heterozygous Pten Y138L mice and between Pten R308C knock in mice (**Table 3.1**). The intercrosses have yielded no live homozygous progeny at birth for the Pten Y138L mutant. It seems likely that this is due to embryonic lethality of Y138L homozygous mice. Instead homozygosity of the Pten R308C mutant transgene did not affect the viability of the embryos. Indeed this analysis reveals a normal mendelian pattern within the R308C offspring composed of 50% heterozygous Pten R308C mice and for 25% each by homozygous Pten R308C mice and WT Pten mice.

Transgenic line		Heterozygous (+/m)	Homozygous mutant (m/m)	Homozygous Wild Type (+/+)	Total
KI Y138L	observed	183	0	72	254
	expected	127	63.5	63.5	
KI R308C	observed	80	45	51	176
	expected	88	44	44	

Tables 3.1|. The genotype of offspring from KI Pten Y138L and KI Pten R308C heterozygous intercrosses was determined by PCR. 1. Expected mendelian ratio and observed genotype in KI Pten Y138L and KI Pten R308C offsprings are shown.

3.1.4 *In vivo* tumorigenesis analysis of KI Pten Y138L and KI Pten R308C mice

It was previously demonstrated that PTEN is a bona fide haploinsufficient TSG, whereas subsequent studies have shown that a decrease of PTEN dosage below normal leads to an accelerated progression to invasive cancer with a variable onset about 8-17 months of age for Pten +/- mice, 12-22 months for Pten hy/+ mice and 5-15 months for Pten hy/- mice (Trotman, Niki et al. 2003), (Alimonti, Carracedo et al. 2010). Pten knock in mice expressing the three mutants PTEN Δ4–5, Pten C124R and Pten G129E alleles display distinct tumor phenotypes in organs targeted by Cowden syndrome with peculiar spectrum of tumours and severity (Wang, Karikomi et al. 2010). Moreover, cancer-related mutations PTEN C124S and PTEN G129E, induce acceleration of tumorigenesis in Knock In mice. They specifically act by further lowering Pten activity rather than engaging alternative pathways. They inhibit the WT protein function in trans in a dominant- negative manner, possibly as a result of heterodimerization. In turn, this reduced Pten lipid-phosphatase activity leads to Akt hyperactivation and increased tumorigenesis in mice. (Di Cristofano, Pesce et al. 1998); (Podsypanina, Ellenson et al. 1999); (Stambolic, Tsao et al. 2000); (Trotman, Niki et al. 2003), (Alimonti, Carracedo et al. 2010); (Wang, Karikomi et al. 2010); Papa, Wan et al. 2014).

To investigate the tumorigenic potential of PTEN Y138L and PTEN R308C mutants, I have established an aging cohort of 60 mice for 6 groups of transgenic mice. These groups are KI Pten Y138L mice WT/YL, KI Pten R308C mice WT/RC KI Pten R308C RC/RC, Pten Null heterozygotes WT/- , Pten Hypomorph heterozygotes WT/Hypo and Wild-Type mice. All the transgenic mice are on C57BL6 background. Mice that do not develop evident tumours are aged until they reached 700 days old, at that point animals are culled and internal organs (including spleen, liver, kidneys, adrenals, uterus, ovaries, prostate, heart, lungs, brain, gut) are analyzed by immunohistochemistry (IHC). If symptoms are identified suggesting hat an

animal has a tumour, they are immediately culled and internal organs are analyzed both in Immunohistochemistry and biochemistry.

Transgenic line	Age of tumourigenic onset (days)	Mean age of alive mice (days)	# Mice dead for tumour (out of 60)	Organ sites of tumours
PTEN WT	580	534	1	Lymph nodes, Gut
PTEN Null het	80-460	-	60	Lymph nodes, Uterus, Intestine, Prostate
PTEN Hypomorph het	620-700	516	3	Lymph nodes, Intestine
KI Y138L het	150-645	473	27	Lymph nodes, Renal, Thymus, Eye, Pancreas, Liver, Gut
KI R308C het	260-644	501	10	Lymph nodes, Gut
KI R308C hom	Unknown	405	2	Lymph nodes, Gut

Table 3.2| Current state of tumourigenic analyses of PTEN Null het, PTEN Hypomorph het, KI Y138L het, KI R308C het and hom mice in comparison with Pten WT expressing mice

The Pten Hypomorphic line is characterized by an insertion of a Neomycin cassette between the exons 3 and 4 that causes a decrease of 40% of Pten expression relative to a single wild-type gene copy. In heterozygosis the amount of Pten expressed in tissues is about 70-80% when compared to Pten WT expressing mice (Trotman, Niki et al. 2003).

I decided to keep the Pten Hypomorphic transgenic line in the in vivo experiments because it represents a good control for our mouse models. Indeed, since even a little reduction in Pten expression could lead to tumour formation, and having showed that the two KI mutants are slightly less expressed than WT Pten, it was important to have a control that could reproduce the slight reduction of Pten expression. In this context, the difference that I have seen between our mouse models and the Pten Hypomorph mouse model gave me the confidence that they were occurring not only because of the reduced expression of KI Pten mutants but mainly because of the partial loss of function of Y138L and R308C, of which I have already speculated above.

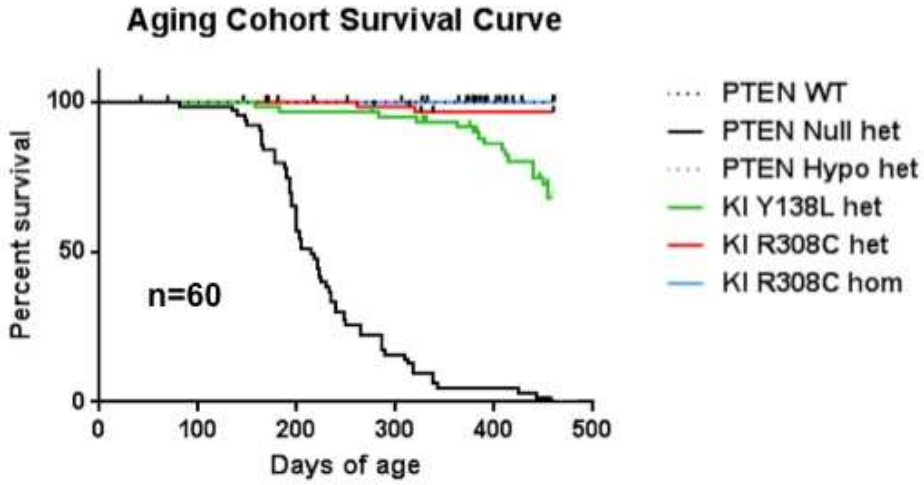

Figure 3.4| Kaplan-Meier plot for percent survival in the aging cohort of the transgenic mouse lines: PTEN WT (Pten WT/WT), PTEN Null het (Pten WT/-), PTEN Hypo het (Pten WT/Hypo), KI Pten Y138L het (Pten WT/YL), KI Pten R308C het (Pten WT/RC) and KI Pten R308C hom (Pten RC/RC). n, number of mice analyzed.

In agreement with published data, 60 out of 60 Pten Null WT/- mice developed spontaneous tumours, mostly lymphadenomas, with an onset between 4 and 13 months old (Trotman, Niki et al. 2003). At the current stage, 27 out of 60 KI Y138L WT/YL mice have developed tumours, suggesting that PTEN Y138L is tumorigenic per se and its expression has implications in tumour formation. Notably, the pattern of tumour formation between the Pten Null and the KI Pten Y138L line appeared to be very different. In the Pten Null line the major site of tumorigenesis were the lymph nodes and only few mice developed tumours in the gut area and in the prostate. Instead, Y138L tumour bearing mice, while having tumours formed in their lymph nodes, they showed also an interesting pattern that was more towards the development of tumours in the gut area, involving organs like intestine, liver and pancreas. Having not seen this peculiar tumour formation pattern in the control transgenic mouse line, this result suggests that it could be related to specific features of Pten Y138L. Instead, currently 10 R308C het mice have developed tumours, mainly involving the lymph nodes and tumours in the gut area, with an onset between 7 and 22 month of age.

The majority of the mice in the Pten Hypomoprhic group have not reached the age that has been described as beginning of tumour growth, previously published by Trotman et al to be as 17-23 months of age (Trotman, Niki et al. 2003). The oncogenic potential of Pten R308C mutant in homozygosis is still under investigation (**Table 3.2**) nevertheless one mouse had developed tumours at the age of 400 days.

3.2 Characterization of KI Pten Y138L

3.2.1 Pten Y138L mutant influences embryos viability

In order to understand at what stage of the pregnancy the Y138L homozygous embryos died, I had stopped the pregnancies and embryos were dissected at the embryo day (E) 8.5, 9.5, 10.5, 12.5 and 16.5 and genotyped those through PCR (**Table 3.2**). No homozygous Pten Y138L embryo was found at the

embryo day (E) 10.5, 12.5 and 16.5, suggesting that the mutation affects the early development of the embryos involving the first 10 days of Instead, when there were genotyped embryos from day (E) 8.5 and 9.5, a few homozygous embryos were found in both the litters (**Table 3.2**). This supported the hypothesis that the homozygous embryos were dieing between E9.5 and 10.5 days of pregnancy. Furthermore, after dissections of litters from E8.5, 9.5 and 10.5, pictures of the embryos were taken by Dr Nadege Poncet. The pictures in Figure 3.5 show embryos of the genotypes Pten WT/WT, WT/Y138L and Y138L/Y138L from different stage of the pregnancy: E8.5, 9.5 and 10.5. The homozygous Y138L embryos were not properly developed in all the stages analysed. Interestingly, at E8.5 embryos were still alive independently from the genotype and E9.5 it was possible to detect heart beating from Pten Y138L/Y138L expressing mice. Instead, homozygous Pten Y138L embryos at E10.5 were not alive. In particular they were unable to properly form a fold that, after the gastrulation period will become the coelemic fold, and later the end of the alimentary canal.

Embryos day	# Litters		Heterozygous (WT/YL)	Homozygous mutant (YL/YL)	Homozygous WT (WT/WT)	Unknown	Total
E8.5	2	observed	11	4	1	0	16
		expected	8	4	4		
E9.5	1	observed	4	5	0	1	10
		expected	5	2.5	2.5		
E10.5	2	observed	9	2	6	0	17
		expected	8.5	4.25	4.25		
E12.5	3	observed	13	0	12	2	27
		expected	13.5	6.75	6.75		
E16.5	2	observed	6	0	9	1	16
		expected	8	4	4		

Table 3.3| Y138L E8.5, E9.5, E10.5, E12.5 and E16.5 were genotyped, expected mendelian ratio and observed genotype in KI Y138L offspring are shown

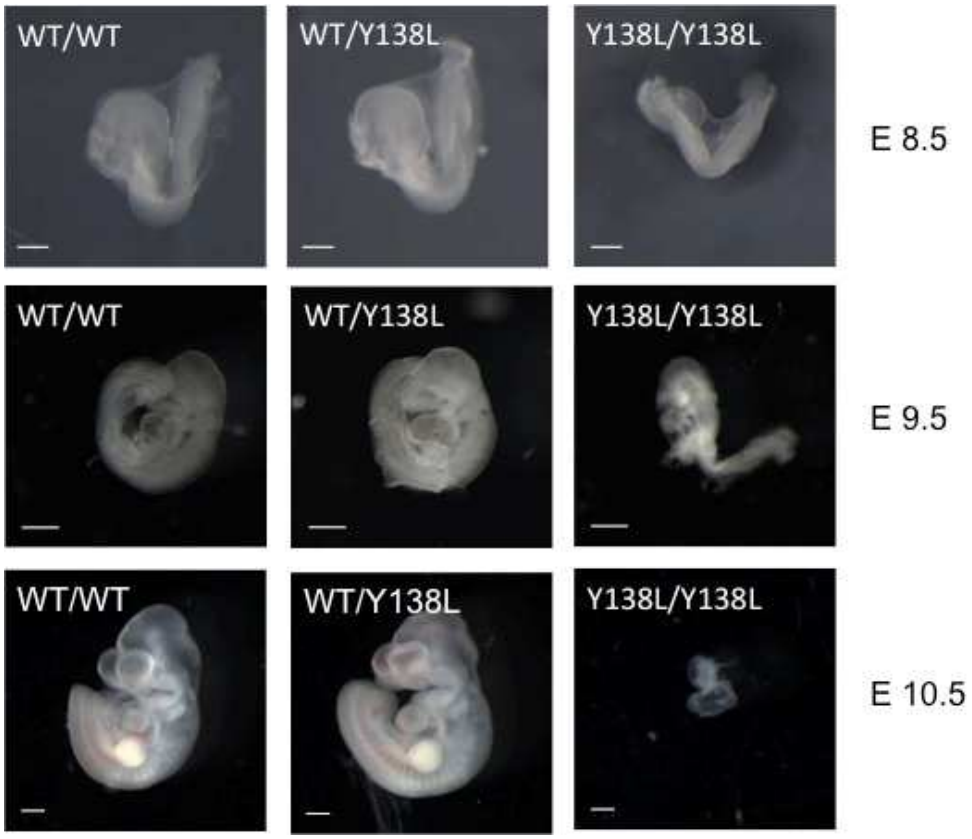

Figure 3.5| Pten Y138L embryos at E8.5, E9.5, E10.5, were dissected out and genotyped. Pictures of embryos were taken post dissections.

In mouse, blastocyst formation occurs at embryo day 4, E4. At E4.5, implantation occurs and the zona pellucida is discarded and the blastocyst attaches to uterine wall. The process of gastrulation begins at about E6.5 in the mouse and by E8 the primitive ectoderm of the post-implantation blastocyst generates the ectoderm, mesoderm, and endoderm of the gastrula. These and other complex processes result in the formation of the tissues and organs that occur in an adult mammal. They require the activation and inactivation of specific genes at specific times, highly integrated cell-cell interactions, and interactions between cells and their non-cellular environment, the extracellular matrix. In general, the embryonic ectoderm gives rise to the following tissues: CNS and outer surface or skin of the organism. The embryonic mesoderm gives rise to skeletal muscles, heart, blood and other connective tissues and the lining of the body cavity. The endoderm, gives rise to the epithelium of the entire digestive tract, epithelium of the respiratory tract, structures associated with the digestive tract (Liver and Pancreas), epithelium of the reproductive ducts and glands. Besides somatic cells, germ cells are also formed during the development of embryo. At E9 there are the first signs of radial asymmetry, during the pre-streak there is an advanced endometrial reaction: the blood starts to invade the ectoplacental cone and the extraembryonic ectoderm and embryonic axis are now becoming visible. By E10, during the Mid-Streak stage the amniotic fold starts to form and by the Late-Streak/Early stage the allantoic bud first appears (Lee, Jeong et al. 2007).

Therefore at E.9.5 and E10.5 during the gastrulation period, which correspond to the Theiler Stages from 14 to 17, there occur the main developmental changes within the embryos that will determine if the embryos would survive and born. It is likely that Y138L mutation may be involved in the disruption of these developmental passages that could determine the lethality of homozygous embryos at E9.5-E10.5. In particular, homozygous Y138L embryos at E8.5 and E9.5 were unable to properly form a fold that, after the gastrulation period will become the coelemic fold, and later the end of the alimentary canal.

3.2.2 Ex-vivo investigation of PI3K /Akt activation Pathway in KI Pten Y138L mice

Understanding PTEN functions that are independent from AKT inhibition has been an understudied area of research. Most of the past studies on PTEN's protein phosphatase activity have relied on the PTEN mutant G129E which selectively retains activity against peptide substrates and lacks significant PtdIns(3,4,5)P_3 phosphatase activity. Using the information from the studies on TPTE and TPIP within a the mutagenic screen, the Tyrosine at the 138 position, essential for PTEN protein phosphatase activity, was mutated to Leucine to get the PTEN Y138L mutant. Phosphatase assays revealed that this mutant lacked detectable activity against polyGluTyr(P) but retained activity against PtdIns(3,4,5)P_3. When expressed in PTEN null cells, PTEN Y138L was capable of reducing the levels of cellular PtdIns(3,4,5)P_3 and phospho-AKT as efficiently as wild type PTEN.

In order to understand if the ability to dephosphorylate lipid substrates and consequently to inhibit Pi3-Kinase/ Akt pathway was maintained in vivo, I decided to analyzed healthy tissues from aged matched young mice (3 males and 3 females from 38 to 42 days old) from the 6 transgenic mice groups. Tissues samples (included brain, liver, heart, thymus, prostate, skeletal muscle and spleen) were dissected snap frozen and lysed in a buffer containing protein phosphatase inhibitor, detergents and reducing agents. Samples from all the tissues of Wild Type Pten expressing mice were first analyzed in Western Blot to optimize blotting conditions and Pten level of expression and activation of Akt and its downstream effector were evaluated (**Figure 3.6**).

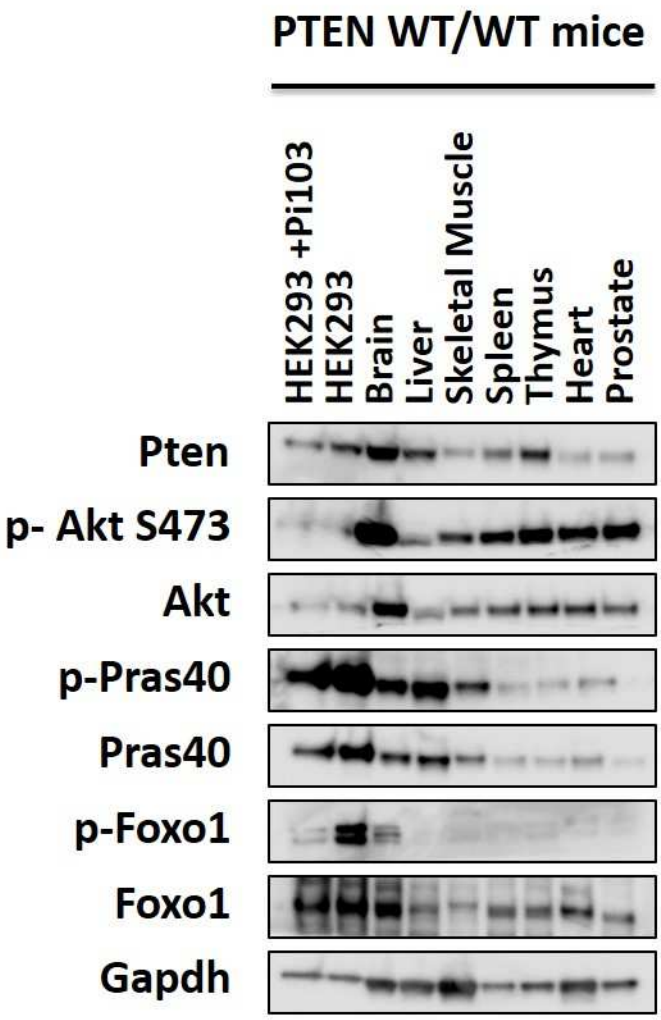

Figure 3.6| Tissues were dissected post-mortem from Pten WT expressing mice. Pten expression, phosphorylation of Akt and downstream targets were investigated by western blotting of brain tissues lysates using total and phospho-specific antibodies.

Next step was to further analyze Pten expression level in other normal tissues and from the results obtained in the experiments described above I choose to study in Western Blot samples from liver and spleen (**Figure3.7**).

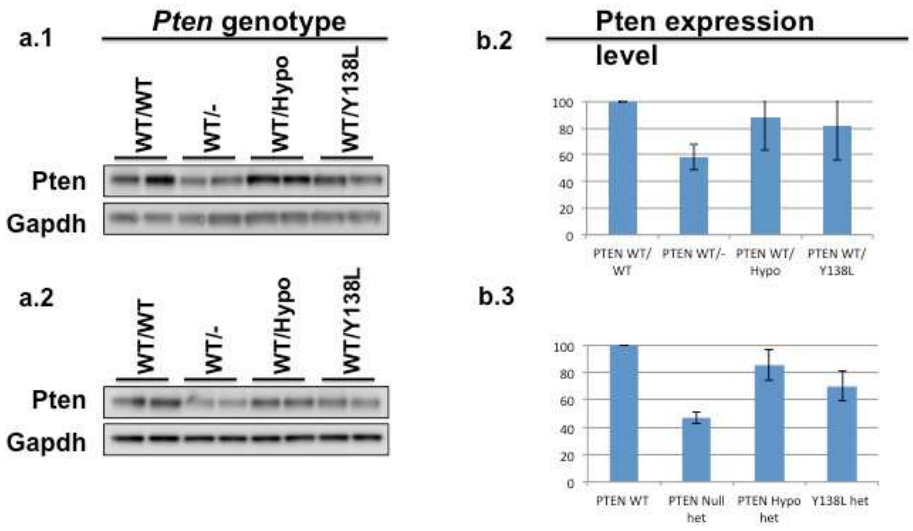

Figure 3.7| a. Healthy tissues (Respectively: a.1 Liver; a.2 Spleen) tissues were dissected post-mortem from Pten WT (WT/WT), Pten Null (-/WT), Pten Hypomorph (Hypo/WT) and KI Pten Y138L (YL/WT). Pten expression was were investigated by western blotting of tissues lysates using specific antibodies. **b.** Percentage of Pten expression in mice pre-neoplastic tissues (Respectively: b.1 Liver; b.2 Spleen) samples analysed in WB related to Pten WT expressing mice. Error bars show STD, n=3.

In the figure is shown one experiment out of 3 per tissue, while the quantification data are related to 3 independent experiments of tissue samples from 2 mice for each group. In agreement with previous shown results, Pten Y138L is slightly less expressed than Pten WT, with a percentage that was about 70-80% in both the preneoplastic tissues liver (**Figure 3.7 a**) and spleen (**Figure 3.7 b**).

Furthermore, aiming to study the activation of Akt and the effectors of the PI-3K pathway in preneoplastic tissues from Pten WT, PTEN -/WT, PTEN Hypomorph WT/Hypo, and KI Pten Y138L WT/YL I have performed Western Blot analysis of brain, liver and spleen from all the transgenic lines listed above.

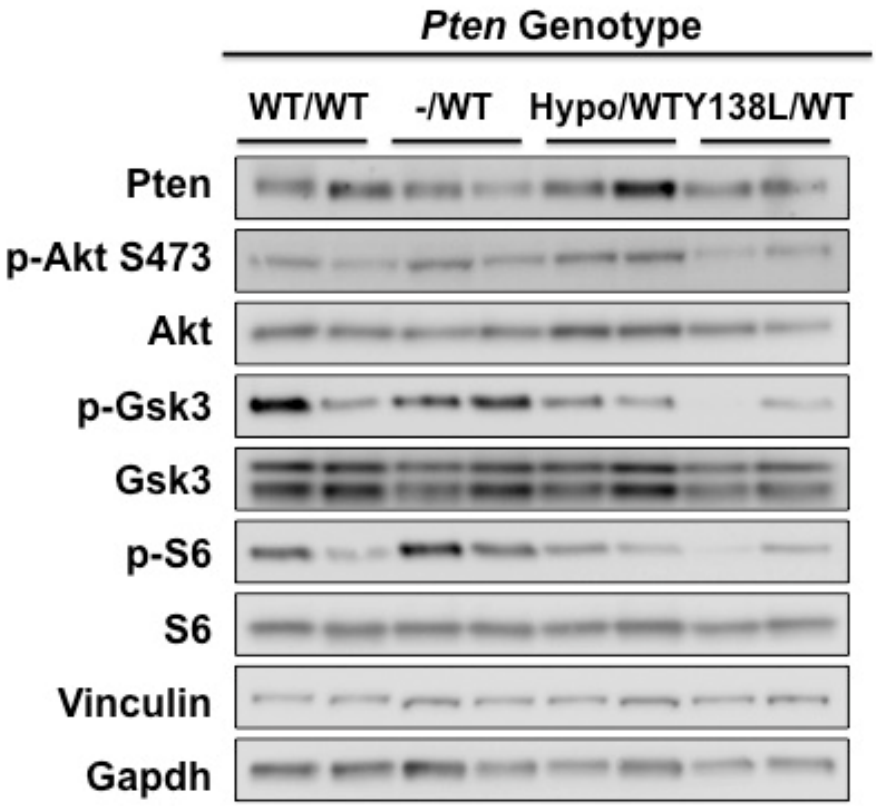

Figure 3.8| Liver tissues were dissected post-mortem from Pten WT (WT/WT), Pten Null (-/WT), Pten Hypomorph (Hypo/WT) and KI Pten Y138L (YL/WT). Pten expression, phosphorylation of Akt and downstream targets were investigated by western blotting of brain tissues lysates using total and phospho-specific antibodies.

Unfortunately, the results were found to be very variable from experiment to experiment. It is already published that the Pten WT/- expressing transgenic mice should be characterized by a higher activation of AKT and its downstream effectors, compared to Pten WT/WT expressing mice Di (Cristofano, Pesce et al. 1998). Instead, in my experiments such increases in the phosphorylation of Akt and S6 were modest or absent. These results are despite having performed all the lysing procedure of the tissue samples on ice using pre-chilled instruments and added a consistent amount of phosphatase inhibitor to the lysis buffer. One of the possible explanations is that the period of time between dissection and freezing of the samples was not short enough to avoid dephosphorylation before freezing and homogenization. Indeed, temperature is one of the most important features that could influence phosphorylation status of proteins. An example of one of these experiments is shown in Figure 3.8, although the patterns in phosphorylation observed were not consistently seen in all experiments.

Despite the extensive analyses conducted, there were no clear results regarding difference of activation or expression of specific downstream effector of Akt among the samples from mice with different Pten genotype. The huge variability between samples expressing the same Pten genotype, due to the difficulty of working with tissues, has limited the interpretation of the data precluding a proper conclusion.

3.2.3 In-vitro investigation of PI3K /Akt activation Pathway in MEFs

Activation of oncogenes or inactivation of tumour suppressor pathways can result in neoplastic transformation. Animal models with altered expression of oncogenes or tumour suppressors have been established to address the role of these genes in tumorigenesis and provide a molecular basis for cancer therapy. Primary mouse embryonic fibroblast (MEF) cells represent a well-defined cell type that has been instrumental in studying the consequences of gene-ablation in cellular growth and proliferation. Moreover, the ability to transform MEFs with oncogenes and their response to growth arrest triggers

has been well characterized. Thus, MEF cells derived from such mutant mice have proven to be an invaluable tool to study immortalization, transformation, and tumorigenesis. (Hong Sun and Reshma Taneja, Cancer genomics and proteomics 2007).

To further analyze the tumourigenic potential of Pten Y138L, stable and immortalized MEF cell lines were derived from Pten transgenic mice by Dr Priyanka Tibarewal in our lab in order to have an in vitro stable transgenic cell model that express only the mutant proteins and allow me to analyze Pten protein function. Including the necessary control MEF cell lines, there were derived MEFs with different Pten genotypes:

- Pten WT/WT (100% expression of WT Pten)
- Pten -/- (0% expression of WT Pten)
- Pten WT/-
- Pten WT/Hypo
- Pten Hypo/Hypo
- Pten WT/Y138L (one allele expressing WT Pten and one expressing Pten Y138L)
- Pten Y138L/- (only one allele expressing Pten Y138L)

MEFs with decreasing dose expression of Pten WT were derived in order to have a control that could mimic the predisposition to tumour development seen in vivo due to a strong but also a subtle reduction of Pten expression (Trotman et al 2003, Alimonti et al 2010). Furthermore, these MEF lines represent also an indispensable tool for study the transgenic mouse lines that are part of the in vivo experiment aimed to analyse the tumorigenic potential of Y138L compared to the two control transgenic mouse lines PTEN Null and PTEN Hypomorph.

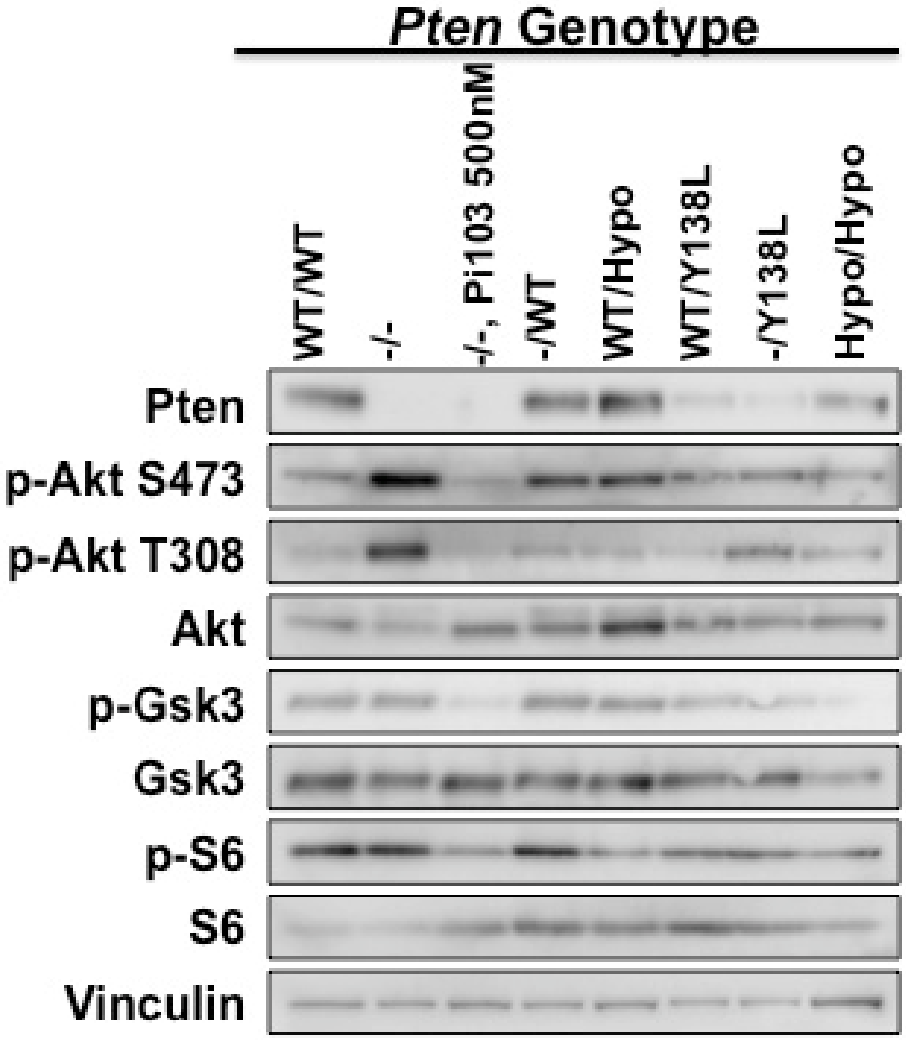

Figure 3.9| Pten WT/WT, -/-, -/- treated with 500nM PI3K inhibitor Pi103, -/WT, WT/Hypo, WT/Y138L, -/Y138L, Hypo/Hypo expressing Mouse Embryo Fibroblassts were analysed in Western Blot. Pten expression, phosphorylation of Akt and downstream targets were investigated by western blotting of tumour lysates using total and phospho-specific antibodies. Pten WT/WT expressing Mefs were used as positive control.

Experiments carried out in the MEF cell lines, showed that the reduction of Pten expression level resulted in an up regulation of Akt phosphorylation, when compared with Pten WT expressing MEF cell line. Interestingly, MEF cell lines expressing 50% of Pten Y138L and 50% of PTEN WT or with only one allele of the mutant were able to control the activation of Akt and its downstream pathway. This result correlate with previous findings obtained in our lab and gave me more confidence that the phenotype show by our mouse model expressing one allele of Y138L is related to an independent pathway from Akt activation and will need more investigation.

3.2.4 *Ex vivo* investigation of PI3K/Akt activation Pathway of Lymphoadenomas developed in mice

Although the PTEN mutant Y138L was identified in a systematic mutagenic screen, it shows the same features as a mutant found in two human cancer samples, PTEN Y138C. This mutant is characterized by the substitution of the Tyrosine at the 138 position with a Cysteine and it was found in a human endometrial carcinoma sample and a lung cancer cell line. These findings suggested that those mutations are cancer-related mutations. Previously described results (**Table 3.2 and figure 3.4**) have revealed clearly the tumorigenic potential of Pten Y138L in mice, indeed it is tumorigenic per se and its expression has implications in tumour formation.

I performed a pilot experiment to investigate Pten expression level and activation of PI3K pathway within the tumours spontaneously developed in mice. I have analysed lysates of two lymphoadenomas grown in Pten Null mice and two in Pten Y138L expressing mice (**Figure 3.10**).

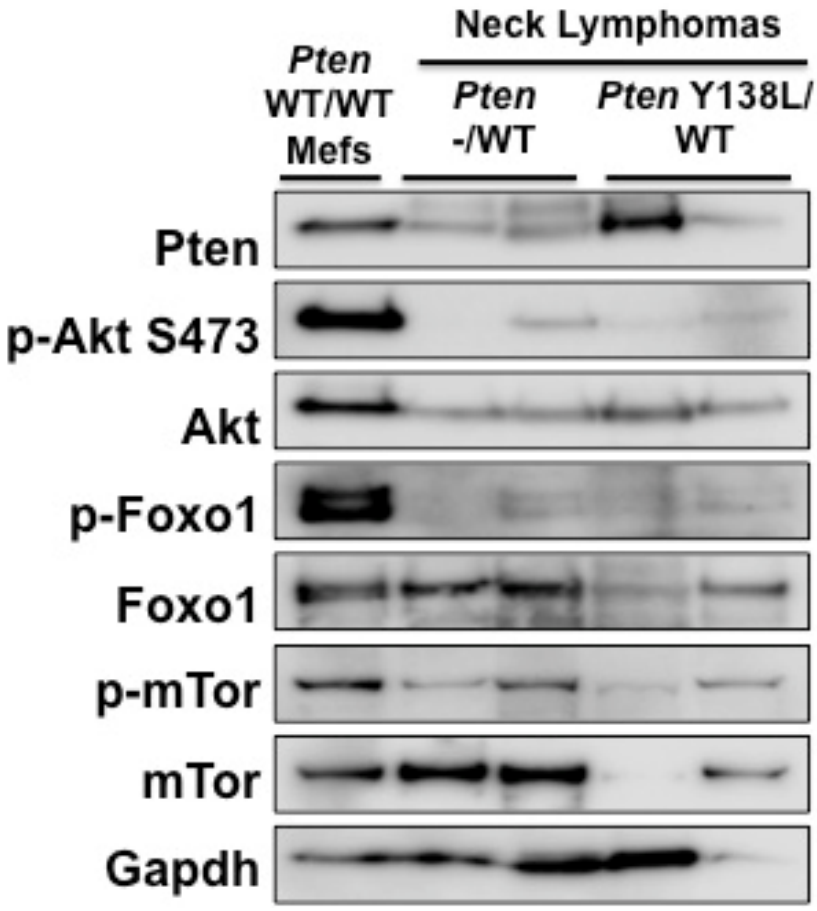

Figure 3.10| Neck Lymphomas were dissected post-mortem from Pten -/WT and YL/WT expressing mice. Pten expression, phosphorylation of Akt and downstream targets were investigated by western blotting of tumour lysates using total and phospho-specific antibodies. Pten WT/WT expressing Mefs were used as positive control.

The results from this experiment are not consistent because it was done only once, but they could reveal that in contrary of Pten Null tumours, in Y138L driven lymphoadenomas there was a better expression of PTEN. In addition, in PTEN Y138L driven tumours, in this single experiment, it is visible a decrease in the expression of total mTOR and FOXO1 compared to Pten WT/- expressing tumours.

3.3 Characterization of KI PTEN R308C

Previous studies in the lab have shown that another mutant, PTEN R308C shared the same features with PTEN Y138L. It's able to control AKT activity and cell migration in vitro but is unable to control epithelial morphogenesis and cellular invasion of U87MG cells. R308C mutation is a missense mutation located in the so called Dloop of PTEN characterized by the substitution of the Arginine at the 308 position with a Cysteine. It was found in two different human tumour samples, a glioma and a malignant uveal carcinoma. It has both lipid and protein phosphatase activity and it didn't affect the stability of the PTEN protein in cultured cells.

At the current stage of our experiment aiming to investigate the tumorigenesis in the KI mouse models, 10 KI Pten R308C het mice have developed tumours, mainly involving the lymph nodes, with an onset between 7 and 22 month of age, while the oncogenic potential of Pten R308C mutant in homozygotes is still under investigation (**Table 3.1**).

Although the tumourigenic potential of this mutant is still under investigation and it does not affect embryo viability, it was important to deeply investigate and uncover a potential phenotype caused by the expression of one or both alleles encoding Pten R308C.

Recent reports on mice with systemic overexpression of the tumour suppressor Pten have expanded our understanding of its physiological functions. Pten over-expressing transgenic mice present increased energy expenditure, decreased adiposity, improved insulin sensitivity upon high-fat

feeding or with aging, and extended lifespan. This has led to new mechanistic insights about the role of PTEN in metabolism. Overexpression of Pten in mice results in reduced body weight, which appears to be a consequence of decreased cell number, while cell size remains intact. Similarly, a study in humans carrying germline mutations in PTEN, and thereby constitutive and systemic decreased PTEN activity, suggested a strong association between decreased PTEN and obesity, although it was also observed a great constitutive insulin sensitization. The observation that PTEN haploinsufficiency can be directly related with obesity in humans, agrees with the finding observed in mice showing that PTEN overexpression protects from obesity. (Ortega-Molina and Serrano 2013).

Considering these recent findings, we considered that an interesting phenotype to investigate could have been the influence by PTEN R308C on mice metabolism and therefore their body weight.

3.3.1 Mice body weight measurement and analysis

To analyse if Pten R308C could influence mice body weight, KI Pten R308C mice expressing WT Pten WT/WT, KI Pten R308C mice WT/R308C, KI R308C R308C/R308C littermates and cage-mates were weighed weekly from 55 to 124 days old (**Figure 3.11**).

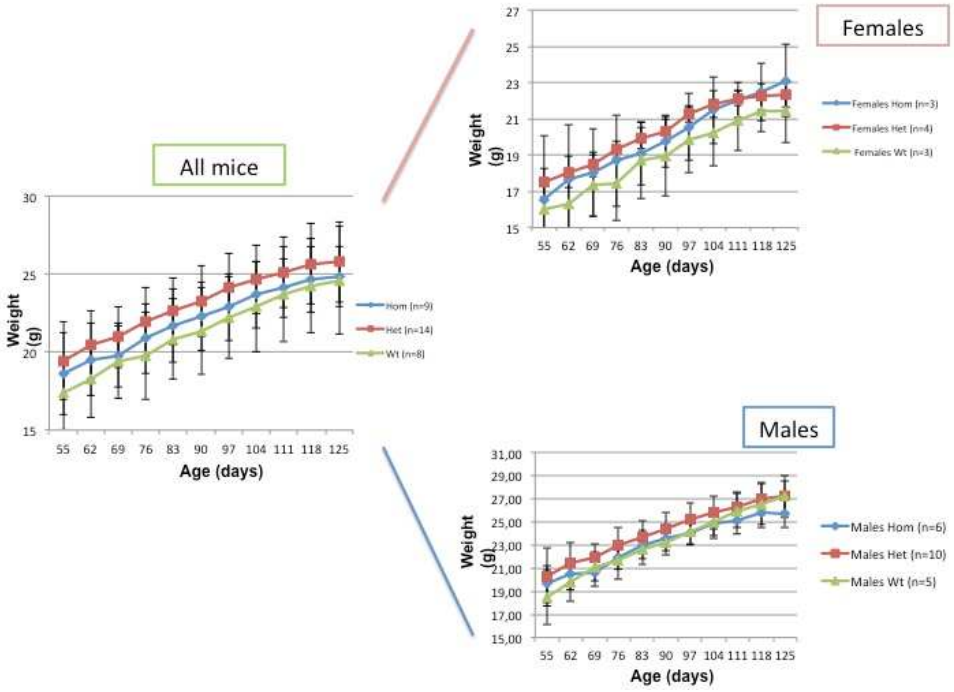

Figure 3.11| Graph showing body weight weekly measurement of KI Pten R308C littermates and cage-mates KI R308C expressing Pten WT/WT (Wt, n= 9), Pten R308C WT/RC (Het, n= 14) and Pten R308C RC/RC (Hom, n= 9) of young mice from 55 to 124 days old. Error bars showing SEM.

Preliminary data suggested that KI Pten R308C heterozygous mice were characterized by an increase in the body weight when compared with Pten WT expressing mice at 55 days old (**Figure 3.11**). Indeed the percentage of variation compared to littermates Pten WT expressing mice was about 15%. However, when the mice were older, it seemed that, while female mice at 124 days old kept being heavier than Pten WT expressing mice, at 124 days in the male mice Pten WT/Y138L mice were almost exactly the same weight as Pten WT mice (**Figure 3.11**).

To clarify this possible effect, I decided then to increase the numbers of the mice that were weighed and analyze the weight of an increased number of with an age between 200 and 300 days old.

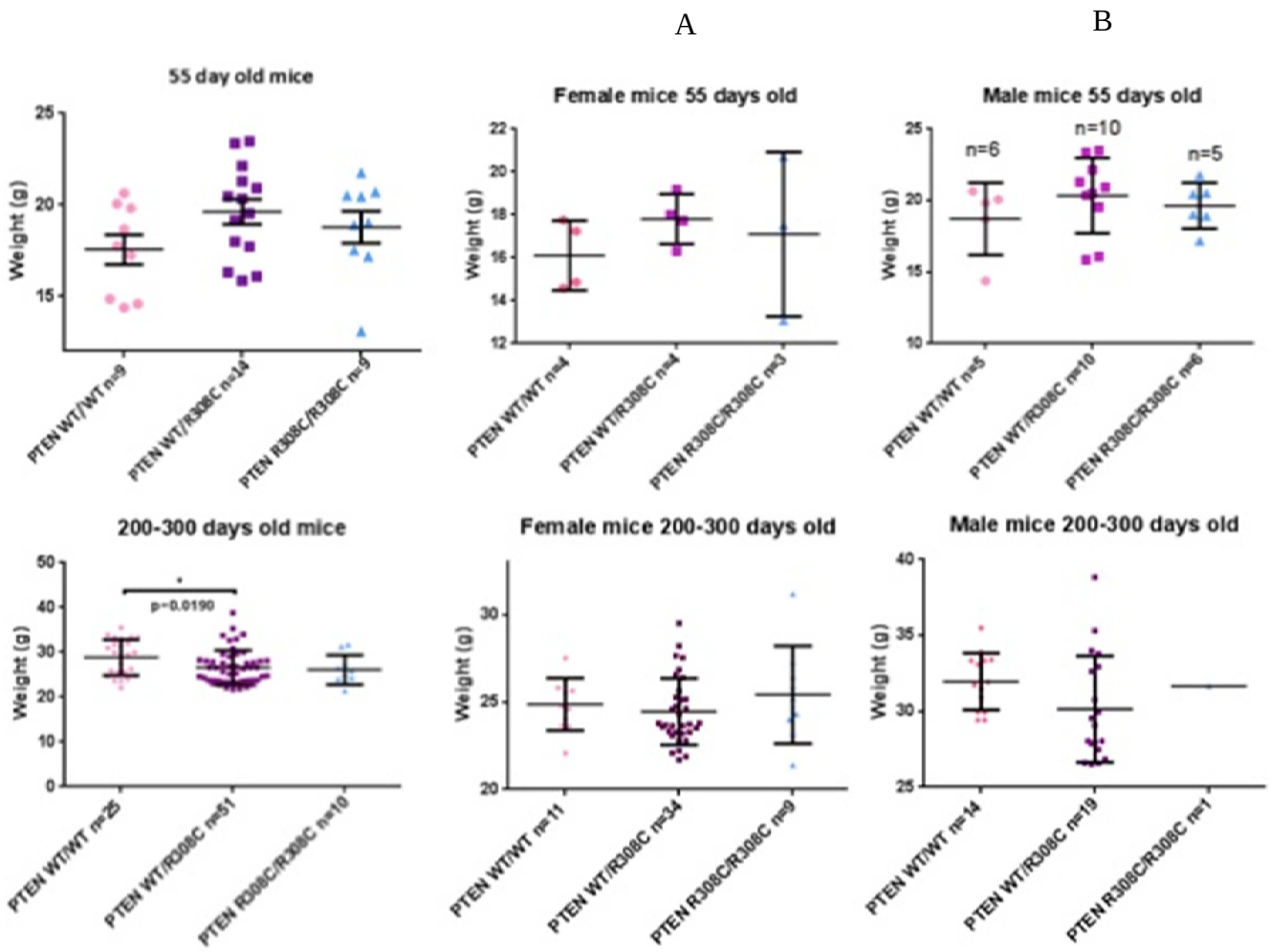

Figure 3.12| **a.** Graph showing body weight measurement of KI Pten R308C littermates and cage-mates KI R308C expressing Pten WT/WT (Wt, n= 9), Pten R308C WT/RC (Het, n= 14) and Pten R308C RC/RC (Hom, n= 9) of young mice 55 days old (a) and older KI R308C expressing Pten WT/WT (Wt, n= 25), Pten R308C WT/RC (Het, n= 51) and Pten R308C RC/RC (Hom, n= 10) mice between 200 and 300 days old (b). Error bars showing SEM.

From this analysis, I could see a decrease in the body weight of homozygous Pten R308C mice compared to those expressing Pten WT. Notably, this result was statistically significant with a p value of 0.019 (**Figure 3.12**) but the significance was not confirmed when the group was split in male and female (**Figure 3.12 A and B**), suggesting that the composition of the groups could influence the results.

3.3.2 Mice adipose tissue deposit measurement and analysis

One factor that could influence mice body weight is the accumulation or depletion of adipose tissue. An increase in the body mass usually reflects a higher adipose tissue accumulation that leads to an increase of body weight. For this reason, I checked if Pten R308C homozygous mice were characterized by depletion in the adipose tissues, including gonadal and retroperitoneal fat. Littermates and cage-mates mice studied as described in figure 3.11 were sacrificed at 200 days old and their fat mass in the gonads and in the area behind the peritoneum was weighed carefully (**Figure 3.13**).

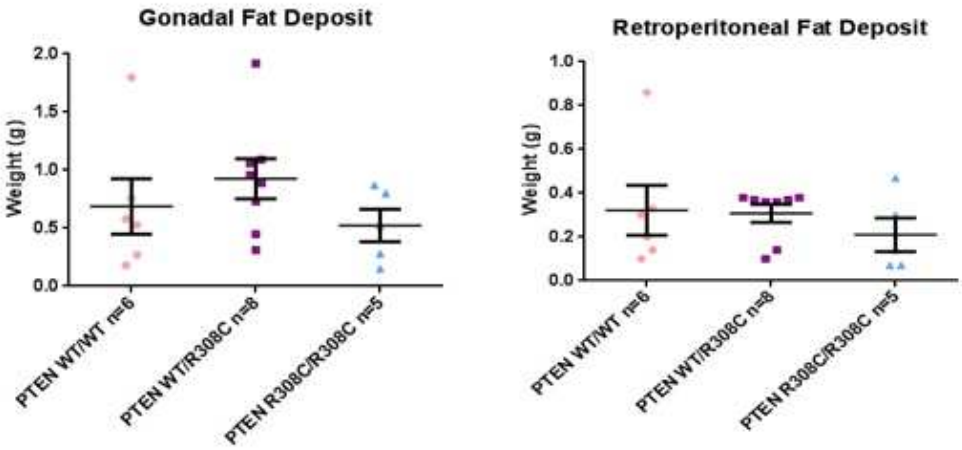

Figure 3.13| Graphs showing adipose deposit measurement of Gonadal Fat and Retroperitoneal Fat of KI Pten R308C expressing Pten WT/WT (Wt, n= 6), Pten R308C WT/RC (Het, n= 8) and Pten R308C RC/RC (Hom, n= 5) of 200 days old mice. Error bars showing SEM.

Both gonadal and retroperitoneal fat seemed reduced in Pten homozygous mice compared to Pten WT expressing mice. Furthermore, the measurement of the gonadal fat might reveal a link between body weight and fat accumulation in the gonads (**Figure 3.13**).

3.3.3 Mice body weight measurement and analysis, a deep investigation

To be confident of the result above, I decided to increase the number of mice analysed. A larger group of mice, including KI Pten R308C mice expressing WT Pten WT/WT, KI Pten R308C mice WT/R308C, KI R308C R308C/R308C of an age between 200 and 300 days old, was weighted once and the data were added to the previous mice studied as in Figure 3.12 **(Figure 3.14)**.

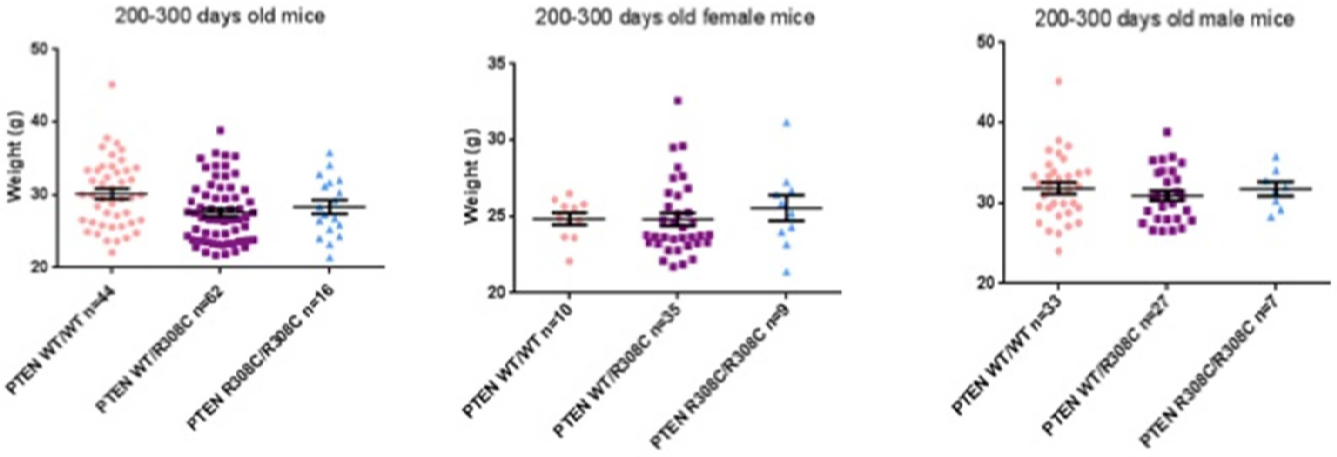

Figure 3.14| a. Graph showing body weight measurement of older KI Pten R308C expressing Pten WT/WT (Wt, n= 44), Pten R308C WT/RC (Het, n= 62) and Pten R308C RC/RC (Hom, n= 16) mice between 200 and 300 days old (b). Error bars showing SEM.

Interestingly, when increasing the number of mice analysed, the result above described was not confirmed. Indeed, the differences among the three genotypes seemed only to reflect a natural variability that is absolutely normal in live animals (**Figure 3.14**). In fact, in this second bigger group the female and male component was more homogeneous. These findings lead me to believe that the R308C mutant may not be involved in a different regulation of body weight compared to Pten WT.

3.4 In vitro study of regulation of gene expression by PTEN through luciferase promoter reporter assay

Regulation of gene expression includes a wide range of mechanisms that are used by cells to increase or decrease the production of specific gene products (protein or RNA). Any step of regulation of gene expression may be modulated, from the DNA-RNA transcription step to post-translational modification of a protein, of which, one of the most important is the transcription initiation. Transcription is initiated at the promoter site, as an increase in the amount of an active transcription factor binds a target DNA sequence. Other proteins, known as "scaffolding proteins", bind other cofactors and hold them in place. DNA sequences far from the point of initiation, known as enhancers, can aid in the assembly of this "transcription machinery."

Gene promoters are typically located upstream of the gene and can have regulatory elements several kilobases away from the transcriptional start site (enhancers). In eukaryotes, the transcriptional complex can cause the DNA to bend back on itself, which allows for placement of regulatory sequences far from the actual site of transcription. Eukaryotic RNA-polymerase-II-dependent promoters can contain a TATA element (consensus sequence TATAAA), which is recognized by the general transcription factor TATA-binding protein (TBP); and a B recognition element (BRE), which is recognized by the general transcription factor TFIIB. The TATA element and BRE typically are located close to the transcriptional start site (typically within 30 to 40 base pairs) (Levine M et al. 2003).

It is not yet known the involvement of PTEN in gene expression regulation. A recent paper published from our laboratory identified a set of transcripts that are induced by PTEN in a manner that does not correlate with AKT. Since they are induced, those gene expression events may make an easily assayable

readout for an AKT-independent signalling pathway regulated by PTEN (Tibarewal, Zidilis et al, 2012 After a deep analyses of the results obtained, I focused my attention on those genes whose expression was more up-regulated only by WT PTEN and not by partial loss of function mutants (**Table 3.4**).

Gene	Modulation of expression
GADD45	Induced by WT PTEN
GDNF	Induced by WT PTEN
VGFR2	Induced by WT PTEN
ILRB2	Induced by WT PTEN
HGMR1	Induced by WT PTEN
FOXO1	Induced by both WT PTEN
WISP2	Induced by both WT PTEN and PTEN Y138L

Table 3.4| List of genes up-regulated by WT PTEN or PTEN Y138L (minimum 5-fold changes) and selected for further luc-reporters analysis. Data are obtained after analyzing microarray output of experiments performed in U87MG cells (Tibarewal et al. 2012).

In order to look at easily assayable integrated biological outputs from multiple signalling inputs downstream of PTEN and in particular to study the less-well-understood signalling mechanisms that correlate with tumour suppression more deeply, I decided to perform luciferase assays based experiments. Through luciferase promoter reporter assay, I wanted to compare the regulation of several genes already recognized as being downstream of the PTEN/ Pi3-Kinase pathway with these recently identified PTEN-regulated transcripts. Luciferase promoter reporters are characterized by the presence of the promoter, or part of it, of the gene of interest fused with the gene that codify for the Firefly Luciferase inserted in a vector that allow the expression of the external DNA in the cells.

Luciferase reporter technology can be a powerful tool for this analysis because it gives unparalleled sensitivity, dynamic range, versatility and ease of use when investigating questions that involve gene regulation. Genetic reporters are used as indicators to study gene expression and cellular events coupled to gene expression.

Luciferase-based genetic reporter assays provide sensitive methods for assaying gene expression, enabling the accurate quantification of small changes in transcription resulting from subtle changes in biology, like pharmacological treatments of cells or expression of specific external proteins.

The response of each gene promoter to the presence of WT PTEN and its mutant or to treatments with the PI 3-kinase inhibitor GDC0941 was deeply analyzed through a luciferase promoter reporter assays. Firstly, U87MG cells, that lack the expression of PTEN, were cultured in both 2D and 3D and transiently co-transfected, when needed, with WT PTEN or PTEN mutants and the luciferase promoter reporter plasmids correspondent to the genes of interest. Most of the luciferase plasmid reporters were kindly provided from scientists who had already used those for their research, while several ones were bought from Addgene. All the gene promoter reporters used are listed in the Material and Methods section (**Table 2.5**).

3.4.1 Evaluation of the specificity of the Dual-Luciferase® Reporter (DLR™) Assay

The DLR™ Assay allows sequential measurement of Firefly luciferase followed by Renilla luciferase activity on addition of Stop & Glo® Reagent to the reaction. Both reporter activities were quantitated within the same sample of lysate prepared from either U87MG or DBTRG cells.

Preliminary experiments were used to optimize the conditions of the assay and to test whether the reporters delivered assayable luciferase activity. HEK293 cells were cultured in adherence and transiently transfected with the luciferase promoter reporter plasmids. After 24 hours of transfection, cells were harvested and lysed and the expressed luciferase activity was measured trough a plate reader luminometer. Preliminary experiments were conducted to optimize the luciferase assays in terms of efficient transfection, optimal concentrations of plasmid DNA, volumes and concentrations of the lysates and setting of the gain for the luminometer used. The ratio between the

plasmid DNA of promoters of interests and the Renilla Reporter Control was kept to 20:1 in order to avoid interference from the Renilla's luminescence signal into the Firefly luciferase's signal and the settings of the lumonometer were set to maintain all the signals from the samples in the linear range of measurement. For some initial experiments, a problem was detected with the Renilla Luciferase plasmid used, seeming to deliver both Firefly and Renilla luciferase channel activities, but which was fixed by a replacement Renilla Luciferase plasmid.

Optimized assay settings were then used to analyze the gene promoters in U87MG and DBTRG gliomas cells and their response to WT PTEN and other mutants of PTEN or the PI3K inhibitor GDC0941.

Firstly, to assess the specificity of the assay and to be confident of the results, I carefully analysed the readout of the measurements of both Firefly and Renilla luciferases that the plate-reader luminometer detected in the samples. I transfected U87MG gliomas cells with 0.5 µM of a luciferase promoter reporter plasmid from the list in table 2.5 in the Material and Methods Chapter that showed robust activity, in the case shown in Figure 4.1 the reporter is the GADD45 promoter reporter gene, or 0.1 µM of the Renilla Luciferase Plasmid. Since Renilla luciferase is under the control of the strong promoter SV40, constitutively active and does not respond to cell environment, it is used in this assay as control for transfections efficiency, I have also co-transfected U87MG cells with both luciferase promoter reporter plasmid and Renilla Luciferase plasmid (**Figure 3.15**). To be used as a transfection control, the Renilla luciferase plasmid includes the strong constitutive promoter SV40 with no evidence of regulated, context dependent transcription. In order to avoid any trans-effect that could influence the specificity of the bioluminescence detected, following manifacturer's instructions, I decided to set a ratio between Firefly Luciferase and Renilla Luciferase of 5:1. Indeed, it is possible to add relatively small quantities of a control reporter vector compared to the reporter plasmids of interest to provide low-level, constitutive expression of that luciferase control activity.

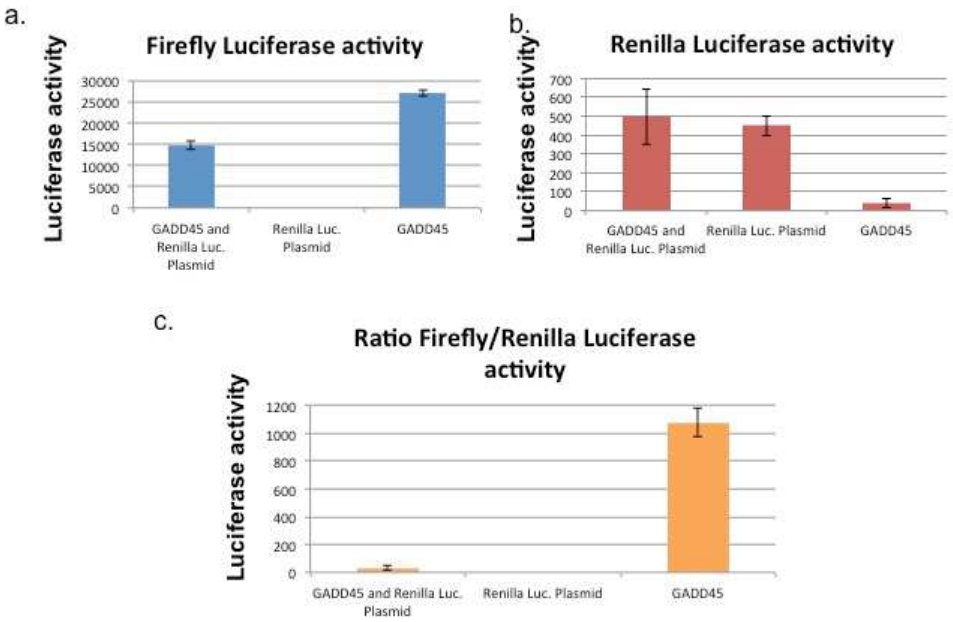

Figure 3.15| The graphs show one representative experiment out of many conducted in U87MG cells. a. Firefly luciferase activity is detected in U87MG cells after 24 hours of transient transfection of the Luciferase Promoter Reporter GADD45 gene and the Renilla Luciferase plasmids together or individually transfected. b. Renilla Luciferase Activity detection in U87MG cells treated as in a. c. Calculation of the Ratio of luciferase activity (Firefly luciferase / Renilla luciferase) in the same samples as in a and b. Error bars show STD, n=3.

This experiment was repeated several times and with different luciferase promoter reporter plasmids. The graph in figure 3.15 is a representative experiment to show that the luciferase activity was specific for both the GADD45 Firefly luciferase reporter and for the Renilla Luciferase plasmid. Indeed, in the samples transfected with the GADD45 gene promoter reporter, only the Firefly luciferase activity was specifically detected (**Figure 3.15 a**) along with a little activity detected also for Renilla luciferase which is probably due to carry over of unquenched Firefly luminescence into the Renilla's measurement wavelength channel. Instead, in the samples in which only the Renilla reporter was introduced, only Renilla luciferase activity could be detected (**Figure 3.15 b**). Furthermore in the samples co-transfected with both the luciferase reporters I could detect both Firefly and Renilla activity and I could calculate the ratio between them (**Figure 3.15**).

These results allowed me to proceed further with the analysis of the gene expression regulation by PTEN firstly in U87MG cells and later in DBTRG using co-transfected Renilla reports as internal controls.

3.4.2 Promoter reporter analysis in U87MG cells

The most known and recognized function of PTEN is counteracting the PI3K-AKT pathway and acting, as a tumour suppressor. Having identified PTEN-responsive genes in microarray expression analysis, we wanted to investigate the mechanisms by which this control is applied. I decided to treat U87MG cells with 1µM of the drug GDC-0941 which is a potent, selective, and orally bioavailable PI3K inhibitor currently in Phase II clinical trials against solid tumours and non-Hodgkin's lymphoma. In particular, GDC-0941 is a potent inhibitor of p110α and p110δ (IC50 = 3 nM) (also less potently p110beta and p110gamma). It selectively binds to PI3K isoforms in an ATP-competitive manner, inhibiting the production of the secondary messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3) and activation of the PI3K/AKT signaling pathway.

Alongside the analysis of the promoters of those genes found in our laboratory to be induced or suppressed by PTEN WT or Y138L, I decided to analyse the activity of the promoter of genes that are already recognized as being downstream of the PTEN/ PI3- Kinase pathway.

U87MG cells were cultured in adherence transiently co-transfected with the Firefly luciferase promoter reporter plasmids together with the Renilla reporter control and after 12 hours of transfection cells were treated with the PI3-Kinase inhibitor GDC-0941. After 18 hours from the treatment, cells were harvested and the luciferase activity of each Firefly luciferase promoter reporter gene in lysates was measured through a plate reader luminometer (**Figure 3.16**).

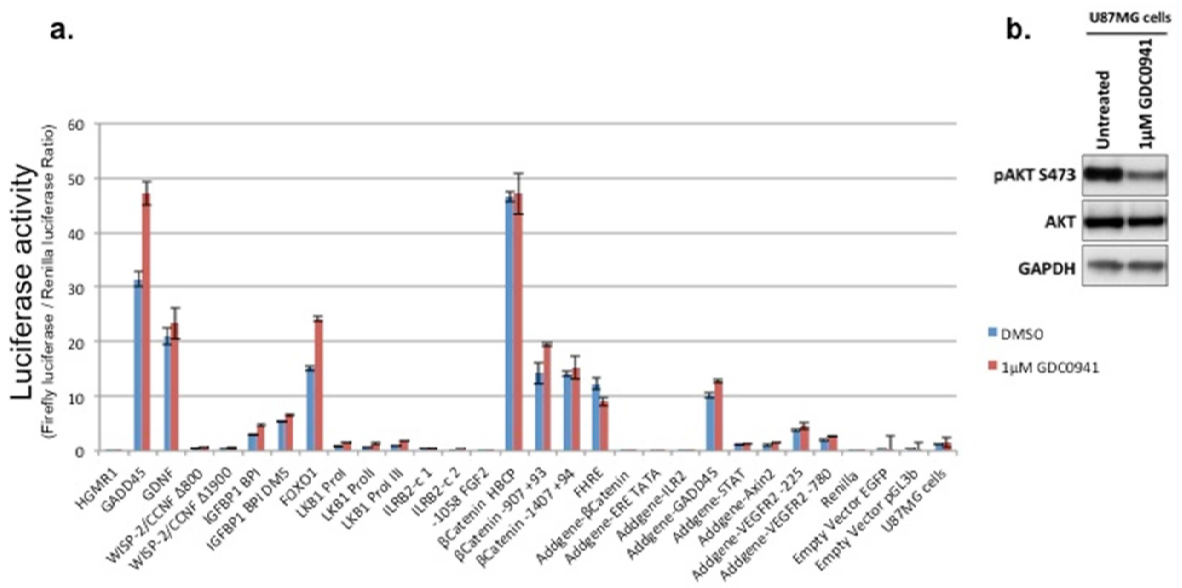

Figure 3.16| a. The graph shows the luciferase activity expressed in U87MG cells after 24 hours of transient co-transfection of different promoter reporter plasmids and the Renilla luciferase reporter plasmid. Cells were treated for 18 hours with 1μM of the PI3K inhibitor GDC0941 or DMSO before performing the assay. The luciferase activity was analyzed using a plate reader that measures the bioluminescence intensity in each sample. Intensity of Renilla Luciferase of each sample was used to normalize Firefly Luciferase activity. Error bars show STD, n=4. b. Western blot of U87MG cells co-transfected as in a. Phosphorylation of AKT and total protein's expression were investigated were investigated by western blotting of cell lysates using total and phosphor-specific antibodies.

26 luciferase reporter fusion constructs were initially tested for their response to PI3K inhibition in U87MG cells. Although some reporters did not have a detectable luciferase activity, the experiment shown in Figure 3.16a revealed that some reporters responded to the treatment with GDC-0941 either by being induced or inhibited. Indeed, the graph shows one representative experiment out of three performed and shows the ratio between Firefly and Renilla luciferase activity of each reporter. In order to confirm that the inhibitor had affected the activation of the PI3K/AKT pathway, cells treated as described above, were lysed and the activation of AKT was analysed in Western Blot. As shown in Figure 3.16b, a decrease is clearly visible in the AKT phosphorylation on the 473 Serine residues in the sample treated with the PI3K inhibitor, compared to untreated cells. This strengthens our conclusion that the activation of the PI3K/AKT pathway was strongly inhibited after 18 hours of treatment with 1µM GDC-0941 and changes in the luciferase activity of the promoter reporter genes were dependent on this.

By dephosphorilating PIP3, PTEN blocks the activation of the PI3K/AKT pathway and from this point of view, the effects could be similar to the treatment of cells with a PI3K inhibitor. For this reason I decided to screen the luciferase reporters also after the expression of PTEN in glial cells lacking of the expression of the tumour suppressor (**Figure 3.17**).

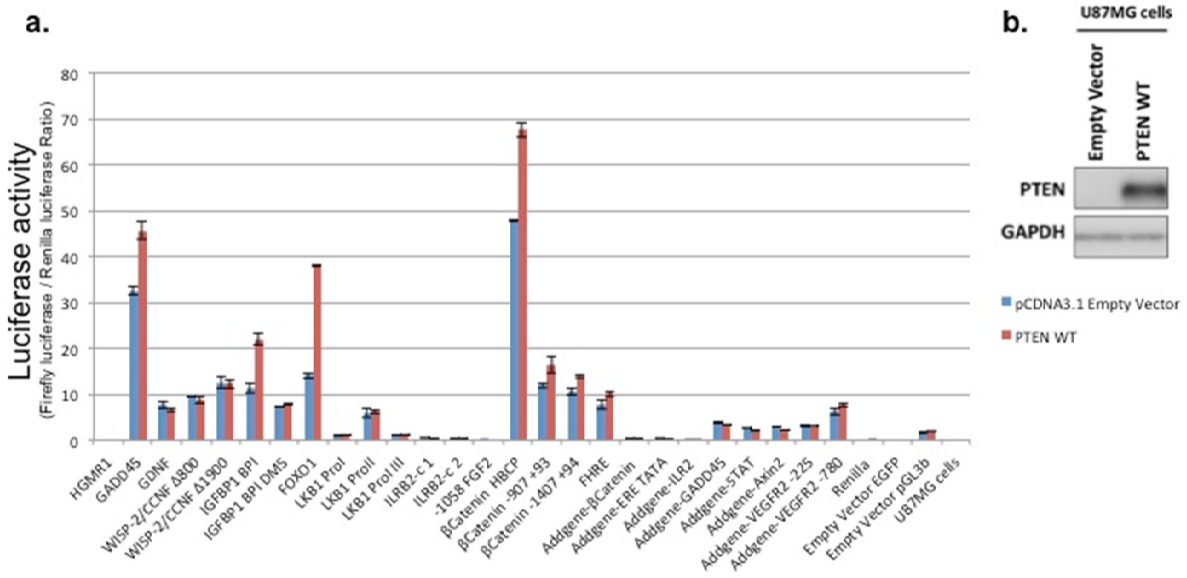

Figure 3.17| The graph shows the luciferase activity expressed in U87MG cells after 24 hours of transient co-transfection of different promoter reporter plasmids and PTEN constructs or pCDNA3.1 Empty Vector and the Renilla Luciferase Reporter Plasmid. The luciferase activity was analyzed using a plate reader that measures the bioluminescence intensity in each sample. Intensity of Renilla Luciferase of each sample was used to normalize Firefly Luciferase activity. Error bars show STD, n=3. . Western Blot of U87MG cells co-transfected as in a. PTEN expression and GAPDH as loading control were investigated by western blotting of cell lysates using specific antibodies.

U87MG cells were cultured in adherence, transiently co-transfected with the Firefly luciferase promoter reporter plasmids together with the Renilla reporter control. In addiction cells were also co-transfected with the empty vector pcDNA3.1 or the vector for the expression of PTEN WT. After 48 hours from the transfection, cells were harvested, lysed and the luciferase activity of each Firefly luciferase promoter reporter gene was measured trough a plate reader luminometer (**Figure 3.17**). In order to verify the presence of PTEN protein after transfection, cells treated as described above were lysed and the expression of PTEN was analysed by Western Blotting. The blot in figure 3.17b shows that PTEN was expressed after the transient transfection; instead cells transfected with the empty vector did not have any PTEN expressed. The graph in figure 3.17 shows one representative experiment out of three performed presenting the ratio between Firefly and Renilla luciferase activity of each reporter.

Although PTEN's function is not limited to the inhibition of the AKT pathway and could affect cellular processes through AKT-independent mechanisms, from the results of this experiments I could discriminate that the effects seen for some of the reporters, after the treatment with GDC-0941, could correlates with the inhibition of AKT. Indeed, some reporters, including GADD45, FOXO1 and IGFBP1 revealed likely correlation with AKT inhibition and made me believe that the luciferase activity assayed in each samples was related to the state of activation of each promoter and their link with the PI3K/AKT pathway.

3.4.3 Assay of selected promoter reporter's activation after expression of PTEN WT and its mutants.

PTEN is constitutively expressed in normal cells but under pathological conditions, PTEN levels can be dramatically reduced, indeed it has been found mutated in many primary and metastatic human cancers. In particular I focused my attention on the functionally selective mutant of interest PTEN Y138L and its control on gene expression compared to PTEN WT.

In order to see if an AKT-independent pathway could regulate the transcriptional regulation of genes of our interest, I wanted to investigate the effects on gene expression of PTEN Y138L compared to PTEN WT and PTEN C124S, which is a catalytic inactive mutant of PTEN. For this analysis I have selected 7 reporters that, in the previous experiment have shown a detectable and specific luciferase activity. I have selected both those whose behaviour appeared PI3K-dependent (like GADD45, FOXO1 and VGFR2) those, instead, who act differently to the treatment with the PI3K inhibitor or the expression of PTEN (like β-Catenin) and those, which not responded to the treatments (like GDNF) (**Figure 3.18**).

In order to verify the presence of PTEN protein after transfection, cells treated as described above were lysed and the expression of PTEN was analysed in Western Blot. The blot in Figure 3.18b shows the expression of PTEN WT and its mutants after 48 hours of transfection.

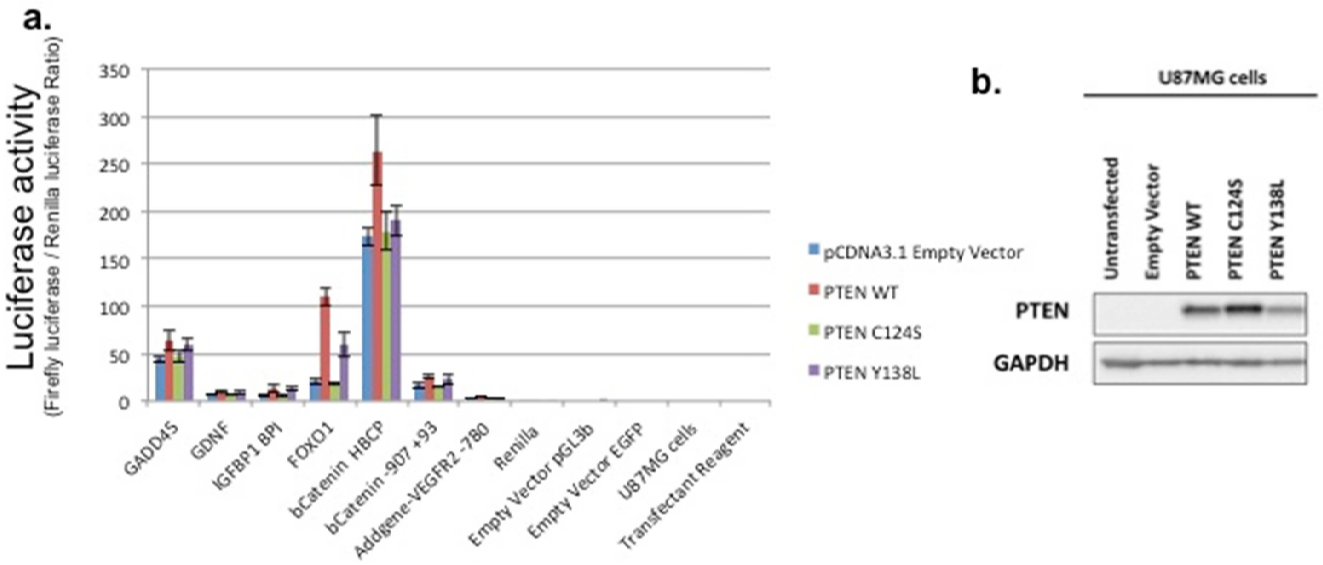

Figure 3.18| A selected representative experiment out of four. a. The graph shows the luciferase activity expressed in U87MG cells after 24 hours of transient co-transfection of selected promoter reporter plasmids and PTEN constructs or pCDNA3.1 empty vector and the Renilla Luciferase Reporter Plasmid. The luciferase activity was analyzed using a plate reader that measures the bioluminescence intensity in each sample. Intensity of Renilla Luciferase of each sample was used to normalize Firefly Luciferase activity. Error bars show STD, n=3. b. Western Blot of U87MG cells co-transfected as in a. PTEN expression and GAPDH as loading control were investigated by western blotting of cell lysates using specific antibodies.

The graph in figure 3.18 show one representative experiment out of four performed in U87MG cells and shows the ratio between Firefly and Renilla luciferase activity of each reporter.

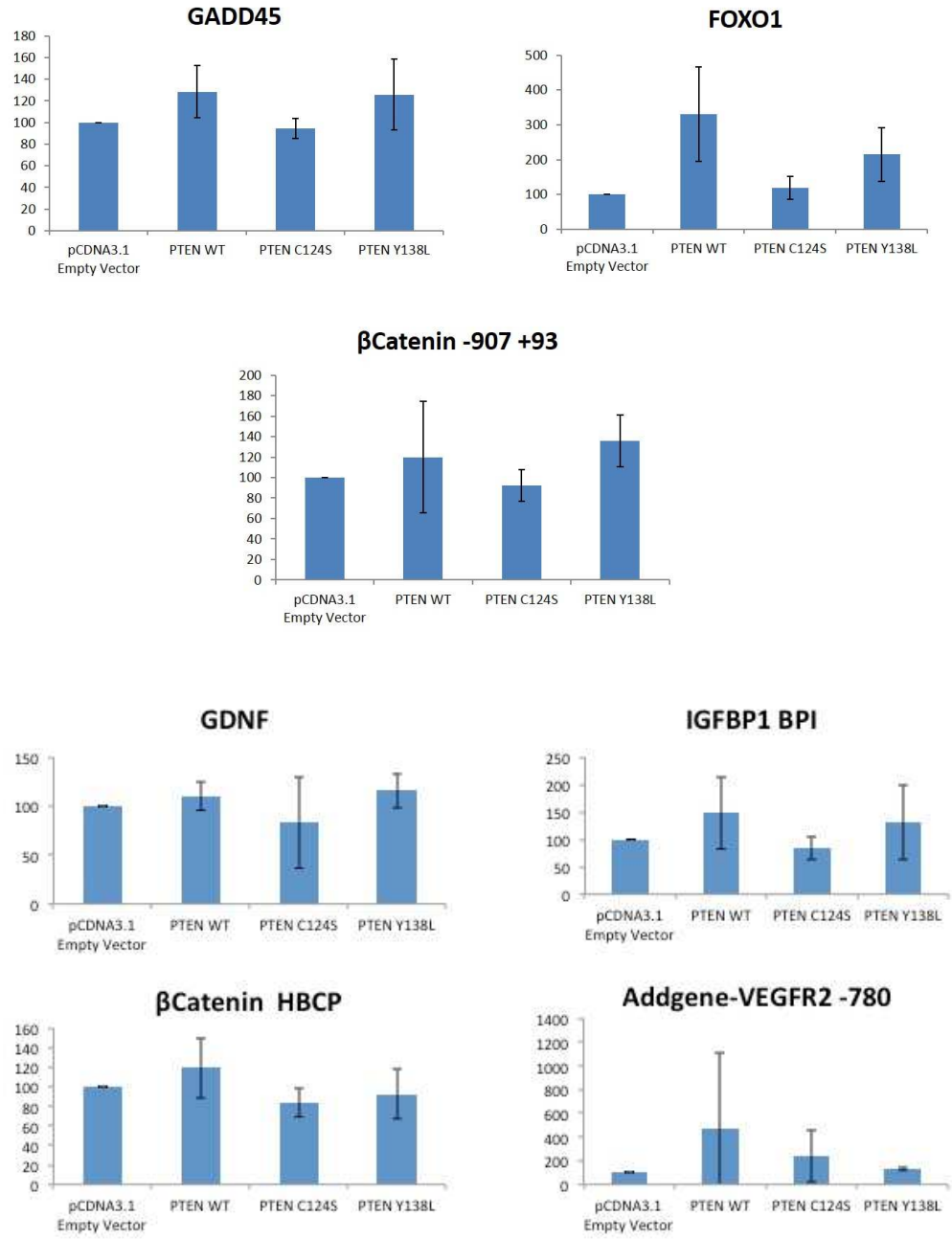

Figure 3.19| T-test analysis of four independent experiments performed in U87MG cells as described in Figure 3.18. P values under 0.0083333 (*) were considered statistically significant. The Bonferroni correction was applied to set P level of significance to set the p value significance threshold. Six multiple comparisons were done. None of the comparisons were found to be statistically different. Error bars show STD, n=4.

I have analysed the means of the luciferase activity of four independent experiments performed for each promoter reporter in multiple comparisons. From this analysis, I observed that the transfection of the empty vector, the catalytic inactive protein PTEN C124S and the catalytic active proteins PTEN WT or the mutant PTEN Y138L did not cause any considerable change in the activation of the reporter genes selected. The combinations that I have done are:

- pCDNA3.1 vs PTEN WT
- pCDNA3.1 vs PTEN C124S
- pCDNA3.1 vs PTEN Y138L
- PTEN WT vs PTENC124S
- PTEN WT vs PTEN Y138L
- PTEN C124S vs PTEN Y138L

For the statistical analyses, I applied the T-test, considering significant a p value less than 0.0083333. Since it included multiple comparisons I have applied the Bonferroni Correction to set the P value significance level. First I have decided a familywise significance threshold (0,05), then I have divided it for the numbers of comparisons that I was making (6). The result of this calculation was 0.0083333.

The analyses revealed that there were no differences between the empty vector pCDNA3.1, the catalytic inactive protein PTEN C124S and the catalytic active protein PTEN WT or the partially active mutant PTEN Y138L (**Figure 3.19**). This could be due to the sensitivity of the test that could not allow differencies among the groups to be revealed.

It is known that FOXOs are key mediators of tumour suppression downstream of PTEN and play a critical role in regulating apoptosis Fu and Tindall (2008), but it is not well known if this regulation could be explained by the regulation of the transcription of the FOXO1 gene and if this process is or is not AKT dependent. To test the hypothesis that PTEN WT and PTEN Y138L could induce the transcriptional activation of the gene encoding FOXO1, I decided to analyse the expression of total FOXO1 protein in U87MG cells transduced

with lentiviruses in order to express PTEN WT or PTEN Y138L in a increasing dose expression. I have chosen to adopt transduction instead of transient transfection because the infection with lentiviruses could allow an efficiency of transduction about 90-100%, while with transient transfection I could only obtained the 30-40% of cells transfected and the percentage of untransfected cells could have influenced the result of the Western Blot by diluting the signal (**Figure 3.20**).

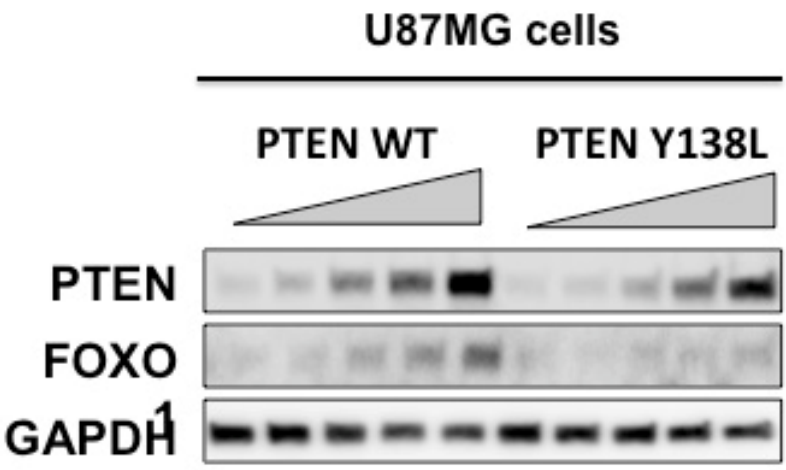

Figure 3.20 Western Blot of U87MG cells transduced with increasing doses of lentiviruses expressing PTEN WT or PTEN Y138L. PTEN, FOXO1 expression and GAPDH as loading control were investigated by western blotting of cell lysates using specific antibodies.

Dr. Priyanka Tibarewal transduced U87MG cells with increasing amounts of lentiviruses for both PTEN WT and PTEN Y138L. Cells were lysed and protein extracts were analysed in western Blot. I have investigated the expression of PTEN along with the expression of total FOXO1. The blot in figure 3.20 shows that when the amount of PTEN protein expressed in the cells was increasing it caused also an increase in expression of FOXO1 in both the samples of cells transduced with PTEN WT and PTEN Y138L. In particular, the increase expression of FOXO1 promoted by PTEN WT was slightly higher compared with PTEN Y138L. It was observed in previous studies in the lab that PTEN Y138L is a less stable protein compared to PTEN WT or PTEN C124S. Because of this issue, I had to be very careful in interpreting the results; the results could also reflect the decrease of the expression of PTEN Y138L and not only its partial loss of function.

It is important to investigate and deep study the activation and the expression's regulation of those proteins in order to fully understand PTEN's functions.

3.4.4 Analysis of transcriptional promoter's activation in 3D cells cultured

Adding the third dimension to a cell's environment creates significant differences in cellular characteristics and behaviour, compared to cells cultured as monolayers on hard surfaces. Importantly, these differences normally create a greater similarity between the cultured cells and the living organism (e.g. human being) the cells are meant to represent – leading to more useful data and more relevant research. In addition, previous studies in our lab had suggested that, culturing PTEN null cells in 2D or 3D cause several changes in cells behaviour including gene expression modifications. For this reason I decided to analyse the activation of the promoter of the genes of interest after 24 hours of 3D culture. For the three dimensional culture I had use a bilayer of matrigel in which U87MG cells were seeded forming the so called "sandwich" and culture medium was put on top in order to give the cells all the nutrients that they needed.

U87MG cells were cultured initially in 2D and transiently co-transfected with the Firefly luciferase promoter reporter plasmids together with the Renilla reporter control. In addiction cells were also co-transfected with the empty vector pcDNA3.1 or the vector for the expression of PTEN WT. After 18 hours from transfection, cells were harvested and transferred in matrigel for 3D culture. Cells were 3D cultured for 24 hours and after this period of time cells were lysed and the luciferase activity of each Firefly luciferase promote reporter gene was measured trough a plate reader luminometer (**Figure 3.21).**

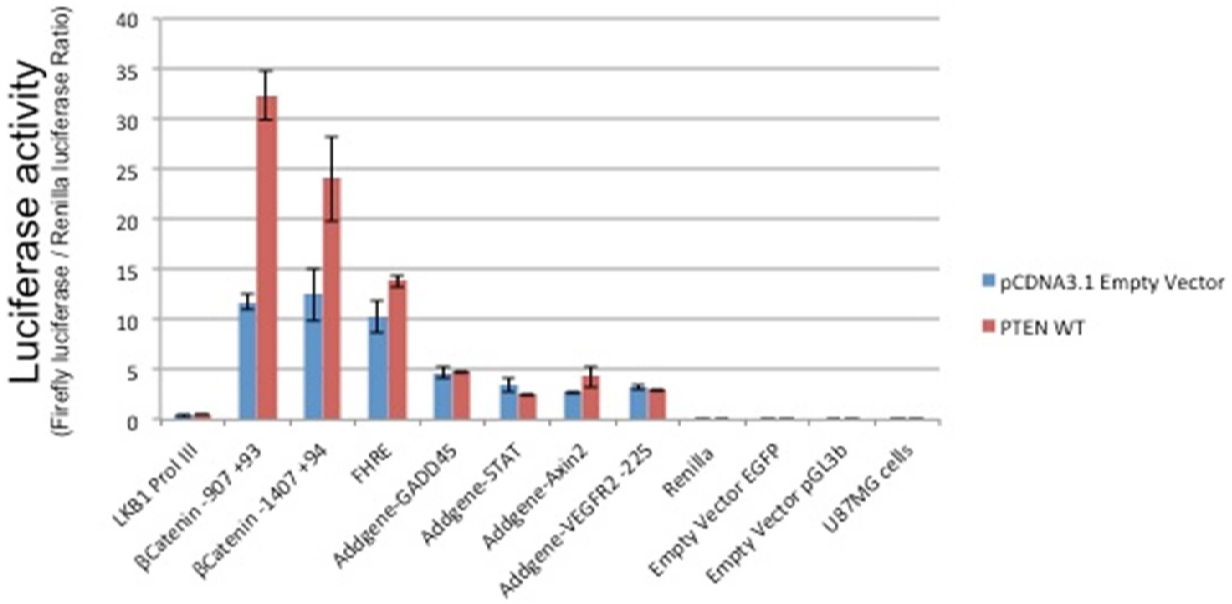

Figure 3.21| The graph shows the luciferase activity expressed in U87MG cells cultured in 3D after 24 hours of transient co-transfection of different promoter reporter plasmids and PTEN WT or pCDNA3.1 Empty Vector and the Renilla Luciferase Reporter Plasmid. The luciferase activity was analyzed using a plate reader that measures the bioluminescence intensity in each sample. Intensity of Renilla Luciferase of each sample was used to normalize Firefly Luciferase activity. Error bars show STD, n=2.

The optimized Dual Luciferase Assay as it was for cells in 2D culture did not detect assayable Firefly or Renilla luciferase activity for all the promoter reporters analysed. Only few promoters had shown a ratio between Firefly and Renilla luciferase that was the result of reliable measurements, including βCatenin (**Figure 3.21**). The graph in figure 3.21 shows one representative experiment out of two performed in 3D cultured cells and bars represent the mean ratio of these promoter reporters. Although the results were not totally clear to be interpreted, it seemed that the activation of the promoters in 3D cultured cells reflected what was revealed from luciferase assays in 2D cultured cells (**Figure 3.17**). From these data, it appears that the activity of these gene promoters may not be dependent on the methodology of culture and so the 3D environment of the cells. It is necessary to better optimize the assay in terms of gain of the laser of the luminometer, concentrations of DNA used for the transfection and number of cells seeded in order to obtain reliable measurements that could be trusted and clearly interpreted.

3.4.5 Screening of the luciferase activity of the gene promoter reporters in response to the inhibition of the PI3K/AKT signalling pathway in DBTRG cells

A screening of the Firefly luciferase activity of the gene promoter reporters assayed in the experiments discussed above was performed using another PTEN null glioma cell line, DBTRG. The main reason for doing that was to validate the results obtained using U87MG cells and the specificity of the Firefly luciferase activity assayed in response to the inhibition of the PI3K/AKT pathway.

DBTRG cells were treated as U87MG cells (**Figure 3.16**). Cells were cultured in 2D, transiently co-transfected with the Firefly luciferase promoter reporter plasmids together with the Renilla reporter control and after 12 hours of transfection cells were treated with the PI3-Kinase inhibitor GDC-0941. After 18 hours of the treatment, cells were harvested, lysed and the luciferase activity of each Firefly luciferase promoter reporter gene was measured trough a plate reader luminometer (**Figure 3.22**).The graph in Figure 3.22 shows an experiment performed in DBTRG cells.

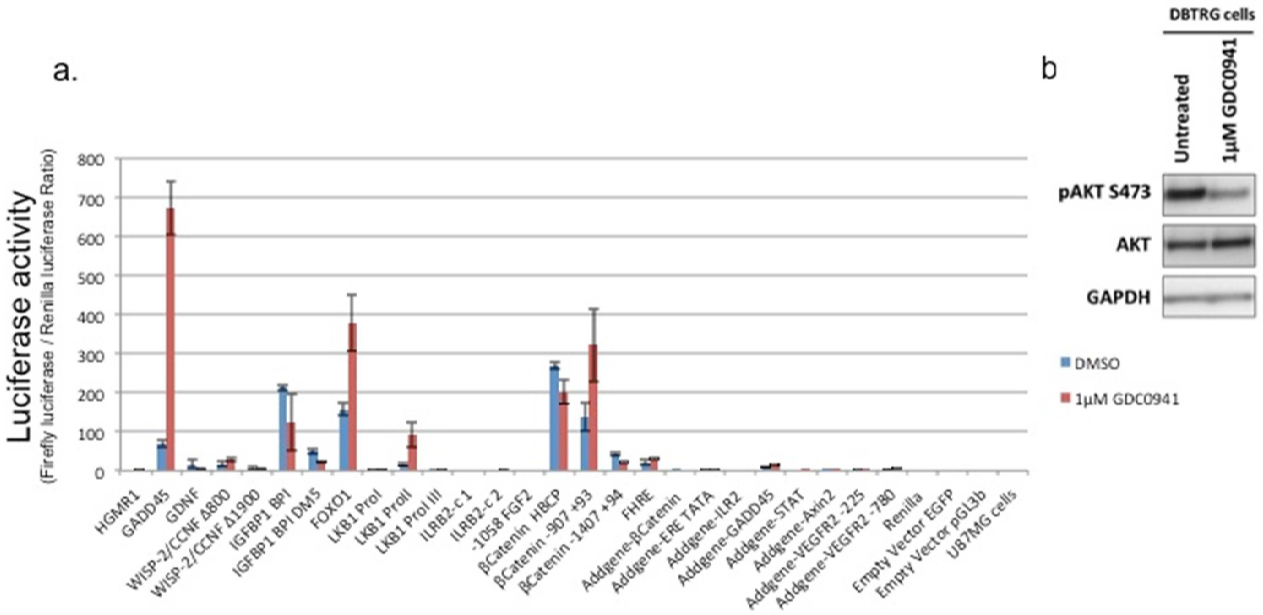

Figure 3.22| The graph shows the luciferase activity expressed in DBTRG cells after 24 hours of transient co-transfection of different promoter reporter plasmids and the Renilla Luciferase reporter plasmid. Cells were treated for 12 hours with 1μM of the PI3K inhibitor GDC0941 or DMSO (Untreated) before performing the assay. The luciferase activity was analyzed using a plate reader that measures the bioluminescence intensity in each sample. Intensity of Renilla Luciferase of each sample was used to normalize Firefly Luciferase activity. Error bars show STD, n=2. b. Western Blot of DBTRG cells co-transfected as in a. Phosphorylation of AKT and total protein's expression were investigated were investigated by western blotting of cell lysates using total and phosphor-specific antibodies.

The same culturing transfection and luciferase assay conditions were used as in the experiments performed in U87MG cells. Unfortunately not for all the gene promoter reporters analysed was detected a reliable value, due probably to the different cell model. Indeed, further optimization steps may be needed in this cell line in order to obtain reliable results as the values of the Renilla luciferase, which is used as control, were not detected uniformly for all the samples and in some cases, Renilla activity was undetectable. This suggest that the ratio between the transfected Firefly luciferase reporter plasmids and the Renilla luciferase plasmid may need to be changed when assaying luciferase activity in DBTRG cells. However, many of the samples provided robust Renilla activity and data for many of the gene reporter seemed to behave as they did in U87MG. This result gave me confidence in the reliability of the findings highlighted above conducted using U87MG cells.

Chapter 4

Conclusions

The aim of this project was to further understand how PTEN regulates tumourigenesis. To do this, two transgenic knock in mice lines were developed expressing Pten Y138L and Pten R308C mutants. I could verify that for both the transgenic mouse line KI Pten Y138L and KI Pten R308C only the missense mutation inserted during the generation of the mice was present (**Figure 3.2**). Therefore, all the analysis and experiments conducted with the new cancer mouse models, both in vivo and ex vivo, allowed me to further understand how Y138L and R308C themselves could influence and regulate tumourigenesis in vivo. Notably, 27 out of 60 KI Pten Y138L WT/YL mice have developed tumours, suggesting that Pten Y138L is tumorigenic per se and its expression has implications in tumour formation. Pten Y138L tumour bearing mice, are characterized by tumours formed in their lymph nodes, which are very common in Pten +/- animals. They also showed an interesting pattern that was more towards the development of tumours in the gut area, involving organs like intestine, liver and pancreas. Having not seen this peculiar tumour formation pattern in the Pten +/- mouse line, this result suggests that it could be related to specific features of Pten Y138L. Instead, currently 10 Pten R308C het mice have developed tumours, mainly involving the lymph nodes, with an onset between 7 and 22 month of age, while the oncogenic potential of Pten R308C mutant in homozygosis is still under investigation (**Table 3.2**).

It is already published that transgenic mouse model lacking Pten, or expressing a reduced [hypomorphic] level of Pten protein or inactive Pten mutants affected homozygous embryos viability. The analyses of the effect of Pten Y138L and Pten R308C on embryonic development revealed that Pten Y138L is embryonic lethal. In particular homozygous embryos died at the E9.5 day of pregnancy. Instead homozygosity of the Pten R308C mutant

transgene did not affect the viability of the embryos (**Table 3.1**). The homozygous Pten Y138L embryos were not properly developed in all the stages analyzed. It is likely that Pten Y138L mutation may be involved in the disruption of these developmental events that could determine the lethality of homozygous embryos at E9.5-E10.5. Indeed, at E9.5 they were not alive and they were unable to properly form a fold that, after the gastrulation period will become the coelemic fold, and later the end of the alimentary canal (**Figure 3.5**).

In order to avoid difficulties due to inter-samples variability of frozen tissue and to study the PI3K pathway activation in mouse, experiments were carried out in the MEF cell lines, showing that the reduction of Pten expression level resulted in an up regulation of Akt phosphorylation, when compared with Pten WT expressing MEF cell line. Interestingly, MEF cell lines with only one allele of the mutant were able to control the activation of Akt and its downstream pathway. This result correlate with previous findings obtained in our lab and gave me more confidence that the phenotype show by our mouse model expressing one allele of Pten Y138L is related to an independent pathway from Akt activation and will need more investigation.

Although the tumourigenic potential of Pten R308C is still under investigation and it does not affect embryo viability, I investigated a potential phenotype caused by the expression of one or both alleles encoding Pten R308C. An interesting phenotype to investigate could have been the influence by Pten R308C on mice metabolism and therefore their body weight. The results obtained lead me to believe that the Pten R308C mutant may not be involved in a different regulation of body weight, compared to Pten WT. In fact, when increasing the number of mice analysed, the differences among the three genotypes seemed only to reflect a natural variability that is absolutely normal in live animals (**Figure 3.14**). It will need more investigation in order to reveal and assess a phenotype that could characterized the in vivo expression of the mutant, either in homozygosis or in heterozygosis.

A better knowledge about these two PTEN mutants, PTEN Y138L and PTEN R308C is therefore critically clinical important. Firstly because the two

mutations are both related to human cancers and secondly because they cause a partial loss of function of PTEN that could not be AKT-dependent. Analyses using these mice should also allow us to understand the mechanisms by which these mutants promote tumour development and test whether we can make predictions about how to match particular patients with drugs to help inform cancer treatment decisions.

In parallel, I have mined gene expression profiling data to investigate how PTEN and Y138L mutant of PTEN regulate gene expression. A recent paper identified a set of transcripts that are induced by PTEN in a manner that does not correlate with AKT. Since they are induced, those gene expression events may make an easily assayable readout for an AKT-independent signalling pathway regulated by PTEN (Tibarewal, Zilidis et al. 2012). Luciferase promoter reporter assays were used to study genes whose transcription is regulated by PTEN. The goal of this latter work is to provide tools to easily reveal AKT-dependent and AKT-independent transcriptional responses controlled by PTEN to investigate mechanisms by which PTEN controls cellular phenotype. Along side the analysis of the promoters of those genes found in our laboratory to be induced or suppressed by PTEN WT or Y138L, I decided to analyze the activity of the promoter of genes that are already recognized as being downstream of the PTEN/PI3-Kinase pathway. Although PTEN's function is not limited to the inhibition of the AKT pathway and could affect cellular processes through AKT-independent mechanisms, from the results of this experiments I could discriminate that the effects seen for some of the reporters, after the treatment with GDC-0941, correlated with the inhibition of AKT.

The reporter that revealed to be interesting was the luciferase reporter of the promoter of FOXO1. In fact, PTEN Y138L was observed to cause a weaker activation of the promoter of FOXO1 compared with PTEN WT (**Figure 3.20**). This result could be due either to the less expression of the mutant or to the partial loss of function of PTEN Y138L and then, to an AKT-independent mechanism. In particular, when the amount of PTEN protein expressed in the cells was increasing it caused also an increase in expression of FOXO1 in both the samples of cells transduced with PTEN WT and PTEN

Y138L. In particular, the increase expression of FOXO1 promoted by PTEN WT was slightly higher compared with PTEN Y138L. Taken together, these findings suggest that PTEN WT is a strong regulator of transcriptional level of FOXO1 and that, PTEN Y138L may have a defect in inducing FOXO1 expression that could be AKT-independent and due to its partial loss of function. This result revealed an important task that should be investigated further.

Both the research projects described in the thesis could help us understanding how PTEN suppresses tumour formation *in vivo* and regulates gene expression in a way not dependent from AKT. These findings will also help inform cancer treatment decisions matching patients with treatments accordingly to their mutational pattern of PTEN.

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